ENDOCRINOLOGY

PUBLISHED FOR

THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

VOLUME 30 MARCH, 1942 NUMBER 3

INTERRELATED VITAMIN REQUIREMENTS: KIDNEY DAMAGE, ADRENAL HEMORRHAGE AND CARDIAC FAILURE CORRELATED WITH INADEQUACY OF PANTOTHENIC ACID[1]

G. C. SUPPLEE, R. C. BENDER AND O. J. KAHLENBERG

WITH THE TECHNICAL ASSISTANCE OF L. C. BABCOCK

From the Borden Company Research Division, Biological and Chemical Laboratories

BAINBRIDGE, NEW YORK

THE RECOGNITION OF PANTOTHENIC ACID as an entity of the vitamin B complex serves to further clarify the identity of vitamin factors essential for life and for maintaining a balanced nutrition. The majority of the studies dealing with this factor as a known variable associate it with growth and prevention of dermatitis in chicks and rats (1–6). It has also been reported to be one of the factors responsible for the cure and prevention of nutritional achromotrichia (7, 8). In addition to these observations, Salmon and Engel (9), Mills et al. (10), Daft et al. (11) and Ashburn (12), have reported severe adrenal disorders manifested by necrosis, fibrosis, hemorrhage, atrophy and depletion of fat in the gland as a result of pantothenic acid deficiency. These data must be interpreted as of special significance in directing attention to a profound pathology directly affecting adrenal secretions and physiological processes dependent thereon. Unpublished investigations carried out at this laboratory not only support many of the above observations but also indicate that the seemingly nonspecific dermal manifestations and exudates encountered in numerous investigations may be syndromes indicative of disordered glandular function resulting from pantothenic acid deficiency.

The potential significance of pantothenic acid as a pre-eminent dietary essential has also been indicated by Supplee et al. (13) wherein mature animals deprived of a then unidentified factor contained in rice polish concentrate exhibited a highly regular reaction pattern characterized by inanition, substantial constant weight for long periods and sudden death without apparent indication of approaching collapse. The present paper records the results from continuing work designed to study these observations more intimately and to determine the status of pantothenic acid and/or other unidentified factors which might be responsible for the phenomena observed.

Received for publication November 26, 1941.

[1] Presented before the American Chemical Society, Division Biological Chemistry, Atlantic City New Jersey, September 11, 1941.

EXPERIMENTAL PROCEDURE

The basal ration used for determining the essentiality of pantothenic acid consisted of vitamin-free casein,[2] 20 parts; sucrose, 69 parts; hydrogenated vegetable oil,[3] 3 parts (or 1, 3 or 10 parts corn oil as noted hereinafter); salt mixture 40,[4] 4 parts; powdered agar-agar, 2 parts; and cod liver oil, 2 parts (or 15 to 20 mg. of halibut liver oil per week). Primary supplements of 12.5 μg. of thiamin, 10 μg. of riboflavin, and 10 μg. of vitamin B_6, all in pure form were supplied as a dosage per rat per day beginning one week after weaning on the unsupplemented basal ration. The variants consisted of synthetic calcium pantothenate and 'rice polish *Factor II*'[5] unheated, or autoclaved at 15 pounds pressure for 5 hours at pH 11–11.5 .White rats 21 to 23 days old and weighing 45 to 50 gm. were used, an equal number of both sexes being included in each test group. Autopsies were made on animals which died during the course of the observation period as well as on those killed at the end of the experiment. Histological examination of the adrenals were made on a substantial number of the positive and negative controls and on animals receiving low calcium pantothenate supplements. Such examination also included tissues from representative animals re-

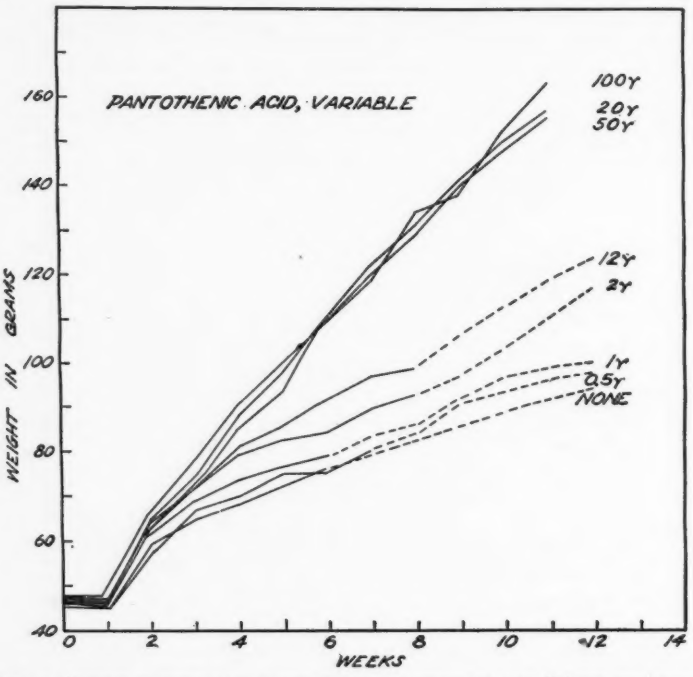


FIG. 1. GROWTH OF WHITE RATS AS INFLUENCED BY PANTOTHENIC ACID. Primary supplements consisted of 12.5 μg. of thiamin; 10 μg. of riboflavin and 10 μg. of vitamin B_6 per rat per day. All animals received the basal ration only for the first week.

[2] Labco Brand, distributed by the Labco Products Department, The Borden Company, New York.
[3] Crisco, Procter and Gamble, Cincinnati, Ohio.
[4] Steenbock, H., and Nelson, E. M., *Jour. Biol. Chem.*, 56: 355. 1923.
[5] Labco Brand, contains no vitamin B_6 or vitamin B_1.

ceiving diets free of one of the following: vitamin B_1, riboflavin, vitamin B_6, and vitamin D. These deficient diets all contained pantothenic acid as the synthetic calcium salt or as present in a natural carrier. Between 250 and 300 animals were involved in the entire study.

Since one of the purposes of this investigation was to correlate the pantothenic acid content of a natural carrier with the animal reaction, it was necessary to establish reliable analytical values for this factor before and after the alkaline heat treatment which is presumed to destroy it. The microbiological method of Pennington, Snell and Williams (14) applied to the unheated rice polish *Factor II* gave values varying from 189 to 216 micrograms of pantothenic acid per gram of solids. After adjusting the pH to 11–11.5 and autoclaving for 5 hours at 15 pounds pressure, values obtained by the same method showed 0 to 3 micrograms per gram for different lots so treated. It may be added parenthetically that rice polish concentrate (desiccated)[6] shows from 180 to 193 μg. per gram before heat treatment and 110 to 133 μg. per gram following alkaline autoclaving when the pH is adjusted to 8.5. It has been determined that the pH of this and many other products decreases during autoclaving; the pH of the alkalinized rice polish concentrate usually decreases to 5.0–5.8.

The growth response of the animals to varying amounts of calcium pantothenate used in the present work is shown in the composite graphs of figure 1. Differences in the growth rate from the zero pantothenic acid level to the 12 microgram level are apparent; however, it is significant that the spread between these extremes is not as great as is obtained from a comparable quantitative supplementation with vitamin B_1, riboflavin or vitamin B_6. The degree of growth at the zero level is greater, however, than is obtained on diets deficient in any one of the factors just mentioned. This seems to confirm the observations of Williams (6) to the effect that it is difficult to deplete weanling animals of pantothenic acid due to its storage in the liver and tissues. A daily supplement of 20, 50 and 100 micrograms of calcium pantothenate revealed no significant difference in growth rate during the 11-week observation period under the controlled dietary and supplement regimen prevailing in these comparisons. The 20-microgram level was supplied as the natural product contained in 100 mg. of unheated rice polish *Factor II*, whereas, the 50 and 100 μg. levels were supplied as the synthetic product.

Numerous deaths occurred at all levels up to 12 micrograms per day especially during the 6th to 10th week; none occurred at the 20-microgram level and above during the 11-week period. All deaths occurred suddenly and without previous significant loss in weight. At the lower levels external symptoms manifested by a 'bloody' incrustation around the eyes and nose was very frequent but not universal. Autopsies revealed adrenal, heart or kidney damage in 60 to 100 per cent of the cases (table 1). The abnormal kidneys were characterized as enlarged, mottled and necrotic. Macroscopically the adrenal damage was described as severe or mildly hemorrhagic. Histological examination, however, clearly revealed extensive hemorrhagic centers, atrophy and necrosis, confined especially to the reticular zone of the cortical layer; the medulla did not appear to be affected.

The occurrence of kidney and adrenal damage was frequently but not always concurrent. Heart damage characterized by enlargement and/or extensive hemorrhage in the auricular region was noted among the animals which had died suddenly on the low pantothenic acid diets, and was frequently concurrent with severe adrenal hemorrhage. A further finding of equal or greater frequency was the high degree of dehydration of the muscular tissue and abdominal cavity. Animals receiving 20, 50

[6] Labco Brand.

and 100 micrograms of calcium pantothenate autopsied at the end of the 11th week and weighing 150 to 160 gm. revealed no evidence of the characteristic pathology shown by the animals receiving 12 micrograms or less. Likewise, none of these characteristic conditions were found in animals receiving the Steenbock rachitogenic diet, or vitamin B_1-free, vitamin B_6-free or riboflavin-free diets.

In order to further correlate pantothenic acid intake with development and maintenance of mature animals, a large group with an equal number of both sexes was supplied the artificial and controlled dietary for a period of 6 months. The primary supplements consisted of 12.5 μg. of thiamin, 10 μg. of riboflavin, 10 μg. of vitamin B_6, and 50 μg. of calcium pantothenate. The variants consisted of 100 mg. quantities

TABLE 1. MORTALITY AND INCIDENCE OF ADRENAL, CARDIAC AND KIDNEY DAMAGE IN YOUNG ANIMALS RECEIVING VARIABLE AMOUNTS OF PANTOTHENIC ACID

(12.5μg. of thiamin; 10μg. of riboflavin; and 10μg. of vitamin B_6 per day as primary supplements)

Calcium pantothenate per day	Mortality		Casualties with adrenal, heart or kidney damage
μg.	%	*weeks*	%
none	43	8	70
0.5	40	9	100
1	88	11	100
2	72	11	100
12	42	8	60
20[1]	none	11	
50	none	11	
100	none	11	

[1] Contained in 100 mg. unheated rice polish *Factor II.*

of unheated rice polish *Factor II* per day containing 20 micrograms of natural pantothenic acid, or 100 mg. per day of the same product alkaline autoclaved at pH 11, containing less than 0.3 μg. of pantothenic acid according to the microbiological method of assay.

The growth and development of the groups of animals receiving each of these supplements is shown in figure 2 (*curves* 1 and 2), in comparison with the growth of the group receiving the primary supplements only, including 50 μg. of calcium pantothenate (*curve* 3). Substantially the same rate of development prevailed in all groups through the 14th week. The rate of growth for the group receiving the primary supplements only, including 50 micrograms of pantothenic acid was not as great from the 14th to 24th week as for *groups* 1 and 2, which received the unheated and alkaline autoclaved rice polish *Factor II*, the supplements containing a total of 70 and 50 μg. of pantothenic acid, respectively.

Ten females were mated from each group at the 14th week with 10 males of the same group. No young were born during a 10-week period in the group receiving the primary supplements only. Two litters were born in each of the groups receiving the heated and unheated rice polish *Factor II* but all the young died within 48 hours due to lactation failure. No deaths occurred in either of the groups receiving the rice polish supplements and only one occurred in the group receiving the primary supplements only. Autopsies at the end of 6 months revealed no evidence of adrenal or heart damage. Slight enlargement and abnormal color of the kidneys were observed in only 2 animals of each group.

Since adrenal damage, particularly of the cortical layer implies impairment of interrelated functions dependent upon the hormone secretions of the cortex and since such functional impairment may precede actual tissue damage, it was desirable to

determine if animals developed on a simplified dietary and rigid control of the vitamin intake with a minimum of pantothenic acid would reflect the effects of the restricted regimen after maturity. Animals were developed to maturity and normal adult weight on the basal ration and usual primary supplements augmented by 100 mg. of unheated rice polish *Factor II* containing 20 micrograms of pantothenic acid. The basal ration

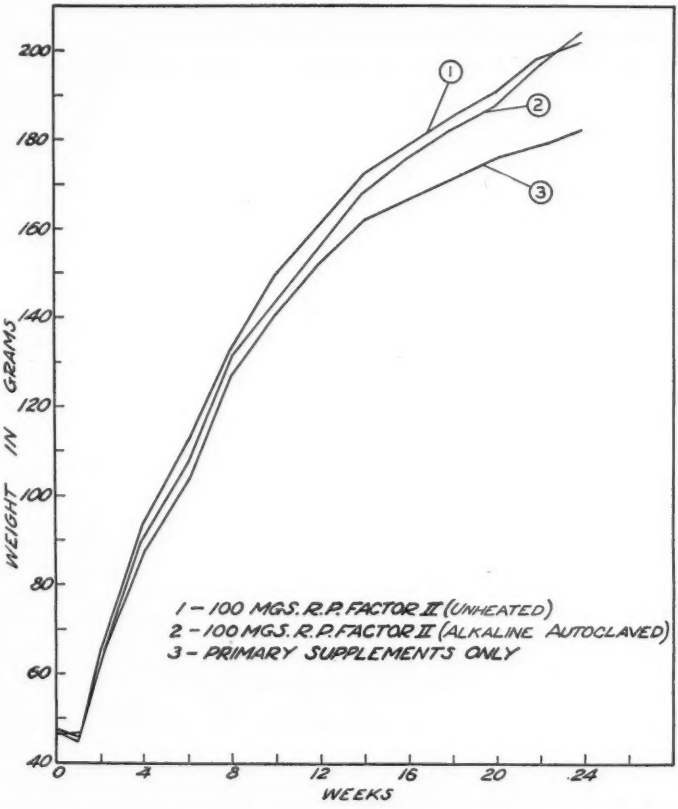


Fig. 2. GROWTH OF WHITE RATS RECEIVING PANTOTHENIC ACID SUPPLEMENTS AS THE SYNTHETIC PRODUCT AND AS CONTAINED IN RICE POLISH FACTOR II. Primary supplements consisted of 12.5 μg. thiamin; 10 μg. riboflavin; 10 μg. vitamin B_6 and 50 μg. of calcium pantothenate per day per rat. All animals received the basal ration only for the first week.

used for this study was modified to contain either no fat, 1, 3 or 10 per cent corn oil in lieu of the 3 per cent hydrogenated vegetable oil; and 15 to 20 mg. per week of halibut liver oil to supply vitamins A and D in lieu of 2 per cent cod liver oil. The supplements were continued through the 11th week with results as shown in the composite graphs of figure 3 from which it will be noted that normal growth was uniformly obtained with the rations containing 1, 3 or 10 per cent corn oil (*curves 2, 3 and 4*); whereas, normal growth did not result from the ration containing no fat (*curve 1*).

From the 11th week through the 15th week the rice polish *Factor II* was with-

drawn from all groups; during this period pantothenic acid was completely absent in the dietary in contrast to the 20 microgram level supplied daily during the initial period of development. Following withdrawal, growth proceeded at substantially the usual rate for about one week, slackened or declined during the second week and rapidly declined thereafter. This reaction is indicative of limited storage of pantothenic acid resulting from the restricted intake during the period of development, but does not necessarily imply functional impairment.

At the end of the 15th week, 50 μg. of synthetic pantothenic acid was supplied per day to all animals. Immediate response in improved growth rate resulted and continued through the 18th week for all groups with the exception of those receiving the no-fat ration. It is considered significant that further growth response did not take place when 100 micrograms per day was provided during the period from the 18th to the 20th week; in fact, all groups remained stationary or declined in weight. Of further significance, however, is the more rapid and continuous decline in weight

TABLE 2. PROGRESSIVE MORTALITY (AS PER CENT) AMONG EXPERIMENTAL ANIMALS AS AFFECTED BY PANTOTHENIC ACID AND PERCENTAGE OF CORN OIL IN THE BASAL RATION

Period *weeks*	Supplement	Corn oil in basal ration			
		none	1%	3%	10%
1–11	Unheated rice polish *Factor II*[1]	0	0	0	0
11–15	None	16	0	0	0
15–18	50 μg. of Ca pantothenate	16	0	0	16
18–20	100 μg. of Ca pantothenate	33	0	0	16
20–24	50 μg. of Ca pantothenate	50	0	0	16
24–35	None	75	50	50	50

[1] Contains 20 μg. of pantothenic acid per 100 mg.

when the pantothenic acid supplement was reduced to 50 micrograms per day during the period from the 20th to the 24th week. This reaction sequence may be indicative of previous functional impairment due to pantothenic acid impoverishment during the period of greatest growth rate or as a result of complete absence from the dietary during the 11th to 15th-week period. Complete withdrawal of pantothenic acid at the end of the 24th week caused a decline in weight in all groups. The mortality rate (table 2) in all groups increased; inanition previous to death and unpredictable collapse was a common characteristic; a 'bloody' incrustation at the nostrils appeared in all groups with equal frequency.

Autopsy findings among the casualties and survivors killed at the end of the 35-week-observation period were characterized by adrenal and/or kidney damage as previously described; heart damage was not observed; dehydration of the tissues and abdominal cavity was especially common. Some of the animals, however, showed none of these conditions. Histological examination of representative specimens of damaged adrenals revealed the same general pathology previously noted for the younger animals.

Richter (15), using the self-selection principle of feeding, has reported that adrenalectomized rats, when offered a choice of water or 3 per cent sodium chloride solution, voluntarily consumed a greater amount of salt as a means of compensating for the normal adrenal function controlling the sodium balance of the body. Adrenalectomized animals having access to salt solution survived for long periods and gained in weight, whereas those animals having access to water only and receiving a dietary containing no salt lost weight and died within a few days following adrenalectomy. Richter concludes that the voluntary increase in salt intake is a natural behavior re-

sulting from 'internal environment,' in this instance absence of the adrenals with obvious impairment of the sodium controlling mechanism. Since the evidence already presented has shown damage to the adrenal cortex resulting from pantothenic acid deficiency, and evidence of physiological dysfunction strongly suggesting impairment of adrenal secretions, it was deemed desirable to determine if presumptive evidence could be obtained in support of the hypothesis that pantothenic acid impoverishment adversely affects the adrenal function concerned with sodium metabolism. An increase in salt consumption by pantothenic-acid-impoverished animals would, in view of Richter's observations and the generally known function of the adrenals in regulating

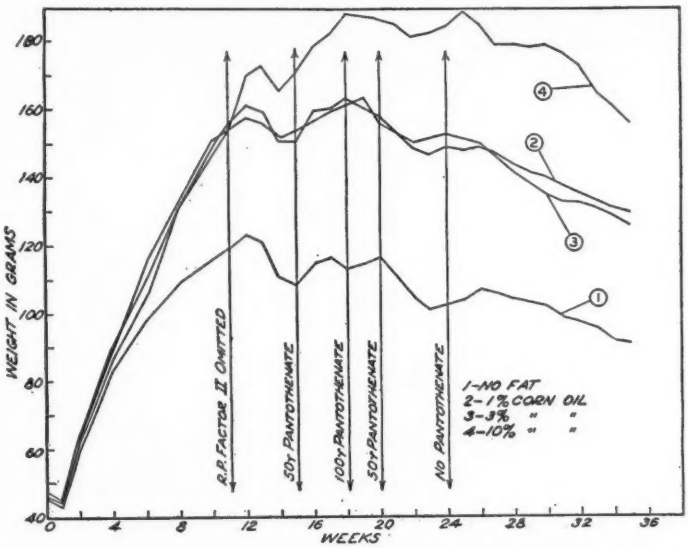


Fig. 3. Growth response and reaction of mature white rats on diets of varying fat content to fluctuating pantothenic acid intake. Primary supplements consisted of 12.5 μg. of thiamin, 10 μg of riboflavin, 10 μg. of vitamin B_6 and 100 mg. rice polish *Factor II* per rat per day for 11 weeks; R.P. *Factor II* omitted thereafter. All animals received the basal ration only for the first week.

sodium metabolism, seem to furnish prima facie evidence of subnormal adrenal function.

Two groups of animals required for the experimental plan were prepared as follows. Both groups received the basal ration containing hydrogenated vegetable oil as heretofore described. The primary supplements consisted of 12.5 μg. of thiamin and 10 μg. of riboflavin per rat per day. *Group 1* also received, in addition to the primary supplements, 100 mg. of rice polish concentrate autoclaved at pH 8.5, per rat per day; when the animals had developed to a weight of about 110 to 120 gm., further increase in weight was suppressed by restricting the vitamin A content of the diet. *Group 2* was supplied with 12 micrograms of synthetic pantothenic acid per rat per day in addition to the primary supplements until the animals had reached a weight of 110 to 120 gm. at which time the pantothenic acid supplement was withdrawn. Each group was maintained at substantially constant weight for a period of some weeks. This was accomplished in *group 1* by reducing the vitamin A supplement while continuing the water soluble factors including the 12 micrograms of panto-

thenic acid, and in *group 2* by withdrawing the pantothenic acid while continuing all other supplements. Both groups were offered water and 3 per cent salt solution at the time constant or substantially constant weight had been established. The amount of water and salt solution consumed by individual animals of each group was recorded daily; food consumption was likewise recorded.

Figure 4 shows in graphical form the comparative daily intake of water and 3 per cent salt solution for representative animals from each of the two groups; the average daily intake of water and salt solution for each entire group as well as the average

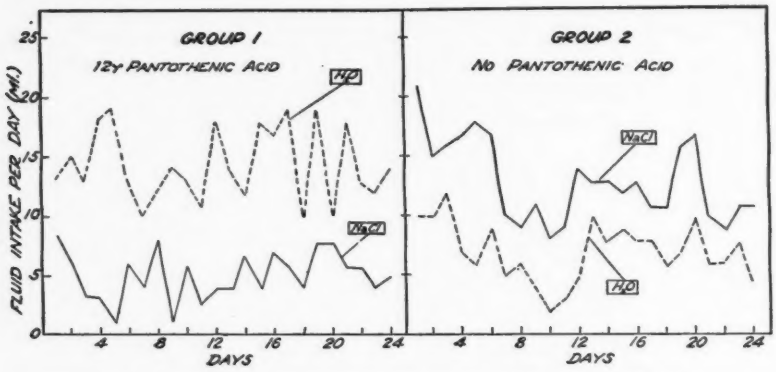


Fig. 4. INFLUENCE OF PANTOTHENIC ACID ON VOLUNTARY CONSUMPTION OF SODIUM CHLORIDE BY WHITE RATS.

Fluid intake per day (Av. of *group 1*)	Fluid intake per day (Av. of *group 2*)
5.1 cc. as 3% NaCl	11.1 cc. as 3% NaCl
16.5 cc. water	9.1 cc. water
21.6 cc. total	20.2 cc. total
Sodium intake per day (Av. of *group 1*)	Sodium intake per day (Av. of *group 2*)
60.1 mg. as 3% NaCl	130.8 mg. as 3% NaCl
12.7 mg. in dry food	11.9 mg. in dry food
72.8 mg. total	142.7 mg. total

daily total intake of sodium, is also shown. The data clearly show a significant and consistently higher sodium intake by the pantothenic-acid-impoverished animals, the average being 142.7 mg. per day in comparison with a 72.8 mg. average for the animals receiving the 12 micrograms daily supplement. Constant or slowly declining weight continued to prevail notwithstanding the increase in sodium intake, and a substantial percentage of the pantothenic-acid-impoverished animals developed the characteristic rust color of the fur on the head, shoulders and back; appearance of the bloody incrustations at the nostrils was not uncommon 4 to 6 weeks after withdrawal of the 12 μg. of pantothenic acid supplement.

COMMENTS

The data presented herein as well as that from other sources seems to show clearly the merits of pantothenic acid for maintaining the integrity of the adrenal cortex. The implications of dysfunction which arise as a result of pantothenic acid deficiency causing cessation or disorganization of the cortical secretions, are manifold and far reaching. All of the functions of the cortex hormones are not thoroughly understood,

some, however, are fairly well established. Sodium metabolism is believed to be controlled by the cortex; insufficiency of cortical secretions result in the elimination of excessive water and sodium chloride causing dehydration and death. Muscular weakness, circulatory collapse, low blood pressure and kidney dysfunction with an increase in urea content of the blood are conditions attributed to cortical deficiencies. Cholesterol metabolism is known to be impaired by adrenal inefficiency, and as a consequence of which, detoxication processes are impaired resulting in excessive fatigue, exhaustion and death. The detoxicating influence of the adrenals is attributed to a cortical hormone rather than to the secretions of the medulla.

The histopathology as observed by Ashburn (12) and as reported herein is apparently confined to the cortex, accordingly it is reasonable to presume impairment of secretion of important cortical substances. Because of the controlling and antagonistic interaction some believe to exist between secretions of medulla and cortex, the effect of unimpaired secretion of adrenalin by the former without the controlling effect of the cortical secretions cannot be predicted; the cardiac hemorrhages and enlargement of the heart noted in conjunction with severe adrenal damage among the animals deprived of pantothenic acid may be significant. The dehydration of tissues and abdominal cavity of animals receiving inadequate pantothenic acid as observed in these studies conforms to certain known effects resulting from adrenal dysfunction. The absence of fat in the damaged cortical layer as observed by Ashburn (12) may be indicative of impaired cholesterol metabolism. Inanition, seeming exhaustion, and unpredictable death as observed in the pantothenic-acid-deficient animals conforms to findings known to result from impaired detoxication processes. Characteristic hemorrhagic degeneration of the kidneys, attributed to choline deficiency, was not recognized as such in this work. While the various diets are obviously low in choline, the reactive methyl contained in the casein compensates in part for this deficiency, and also the unheated rice polish *Factor II* carries 700 micrograms of choline per 100 mg. quantity. It is believed, therefore, that the renal damage noted is primarily attributable to the effects of pantothenic acid deficiency rather than to choline deficiency.

SUMMARY

Pantothenic acid appears to be required for maintaining the integrity of the adrenal cortex. Its absence or inadequacy in experimental diets results in a characteristic pathology of the cortical layer readily detectable by macroscopic and histological examination.

Pantothenic acid influences growth rate but seemingly not in a direct arithmetical relationship. Twelve micrograms or less of calcium pantothenate per rat per day retarded growth and led to numerous sudden deaths within 8 to 10 weeks. Twenty micrograms and above permitted normal growth when fed in conjunction with other synthetic vitamin supplements.

Pantothenic acid deficiency in white rats is characterized by slow growth rate, inanition suggestive of exhaustion, and sudden death without objective symptoms of impending collapse. Autopsies in the great majority of cases revealed kidney, adrenal or cardiac damage, and with equal or greater frequency, excessive dehydration of muscle tissue and abdominal cavity.

Mature animals deprived of pantothenic acid for about 4 weeks exhibited a latent reaction which is interpreted as evidence of serious or permanent functional impairment.

Mature animals deprived of pantothenic acid following a relatively low pantothenic acid intake during development voluntarily consumed about twice as much

sodium as sodium chloride solution as did animals of the same weight not deprived of pantothenic acid; this evidence is indicative of adrenal function impairment resulting from pantothenic acid impoverishment.

The data as a whole indicates the specificity of pantothenic acid for maintaining a normal balance of adrenal functions; the quantitative requirement by the individual organism is seemingly determined by an interrelationship of factors, including dietary components and vitamin intake, state of development and maturity, and previous physiological history.

REFERENCES

1. Jukes, T. H.: *J. Am. Soc.* 61: 975. 1939.
2. Oleson, J. J., D. W. Woolley and C. A. Elvehjem: *Proc. Soc. Exp. Biol. & Med.* 42: 151. 1939.
3. Unna, K.: *J. Nutrition* 20: 565. 1940.
4. Unna, K., and W. L. Sampson: *Proc. Soc. Exp. Biol. & Med.* 43: 309. 1940.
5. Richardson, L. R., and A. G. Hogan: *Proc. Soc. Exp. Biol. & Med.* 44: 583. 1940.
6. Williams, R. J.: *J. Nutrition* 19: 7. 1940.
7. György, P., and C. E. Poling: *Science* 92: 202. 1940; *Proc. Soc. Exp. Biol. & Med.* 45: 773. 1940.
8. Frost, D. V., R. C. Moore and F. P. Dann: *Proc. Soc. Exp. Biol. & Med.* 46: 507. 1941.
9. Salmon, W. D., and R. W. Engel: *Proc. Soc. Exp. Biol. & Med.* 45: 621. 1940.
10. Mills, R. C., J. H. Shaw, C. A. Elvehjem and P. H. Phillips: *Proc. Soc. Exp. Biol. & Med.* 45: 482, 1940.
11. Daft, F. S., W. H. Sebrell, S. H. Babcock, Jr. and T. H. Jukes: *U. S. Pub. Health Repts.* 55: 1333. 1940.
12. Ashburn, L. L.: *U. S. Pub. Health Repts.* 55: 1337. 1940.
13. Supplee, G. C., R. C. Bender and O. J. Kahlenberg: *J. Nutrition* 20: 109. 1940.
14. Pennington, D., E. E. Snell and R. J. Williams: *J. Biol. Chem.* 135: 213. 1940.
15. Richter, C. P.: *Am. Jour. Psychol.* 97: 878. 1941.

THE EFFECT OF DESOXYCORTICOSTERONE ACETATE ON THE BLOOD PRESSURE OF THE DOG[1]

S. RODBARD AND S. C. FREED

From the Cardiovascular Department and the Department of Medicine, Michael Reese Hospital

CHICAGO, ILLINOIS

HYPERTENSION HAS BEEN OBSERVED in patients with Addison's disease receiving overdoses of desoxycorticosterone acetate (1–4). This has also been reported in adrenalectomized (5) and in normal (5, 6, 7) animals. The present study was undertaken to determine whether a like pressor action could be induced in dogs already hypertensive.

METHOD

Blood pressures were recorded every 3 to 7 days on 12 trained unanesthetized dogs, using the Hamilton manometer (8). Hypertension was produced by constriction of the renal arteries with linen ligatures. Desoxycorticosterone acetate (20 mg. daily) in sesame oil[2] was injected subcutaneously at approximately the same time each day. The blood total chlorides, cholesterol, cholesterol esters, creatinine, inorganic phosphate, sodium, calcium, potassium, nonprotein nitrogen and glucose were determined at varying intervals.[3]

RESULTS

Desoxycorticosterone acetate was given subcutaneously to 6 dogs with hypertension due to partial constriction of the main renal arteries. These results were compared with those on 3 normotensive and 3 spontaneously hypertensive dogs. The results are summarized in table 1; the values shown are typical of the effects in each animal. The blood pressure changes on one of the hypertensive and two normotensive dogs are shown in figures 1 to 3.

The effect of these massive doses of desoxycorticosterone acetate upon 6 Goldblatt-hypertensive dogs (Y41, X83, X8, X61, X54 and Y103) was to elevate the blood pressure to higher levels in 5 of them. In the 6th (X8) the blood pressure was unaffected. In two of these dogs the development of pulmonary edema led to death at the end of the 3rd week of injections of desoxycorticosterone acetate (X54, X61). In one of the dogs that survived, cessation of desoxycorticosterone acetate administration led to a fall in blood pressure to its preexisting hypertensive level (X83).

Of the 6 normotensive and spontaneously hypertensive animals, a rise in blood pressure was seen in 4 (X49, W76, Y1 and X34). In the other two dogs no such elevation occurred (X46, W77). In two animals a temporary initial blood pressure fall occurred (W76 and W77). The rise in blood pressure persisted for 24, 40 and 62

Received for publication December 18, 1941.

[1] Aided by the A. D. Nast Fund for Cardiac Research and the Isaac and Kate Meyer Fund.

[2] We are grateful to Dr. Max Gilbert of Schering Corporation, Bloomfield, N. J., and to Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Summit, N. J., for generously supplying the desoxycorticosterone acetate used in this investigation.

[3] We are grateful to Dr. D. J. Cohn for his cooperation in having these determinations made in the Department of Chemistry.

days respectively, after desoxycorticosterone acetate injections were discontinued in 3 of the animals; in the other dog the blood pressure quickly returned to normal levels (X49).

Cholesterol, cholesterol esters, creatinine and chlorides of the blood showed no significant variations following administration of desoxycorticosterone acetate nor

TABLE 1. EFFECT OF DESOXYCORTICOSTERONE ACETATE ON BLOOD PRESSURE

Dog No. and sex	Wt.	Previous intervention[1]	Dosage	Duration of administration	Values during injection desoxyacetate					Results after stopping treatment
					Day	Blood pressure		Serum K	State of animal	
						systolic	diastolic			
	kg.		mg./kg./day	days		mm. Hg	mm. Hg	m.eq./l.		
X46, ♂	17	Spontaneous hypertensive	0.5	46	0	190	110			Died on 46th day of administration; organs normal at autopsy
					37	185	115			
W77, ♂	15	Normotensive	0.6	72	0	150	75	5.4		Spasticity disappeared in 2 days
					20	120	65			
					40	130	70	2.3	Weak	
					54	145	75	2.6	Spas. mus.[3]	
X49, ♂	15	Spontaneous hypertensive	0.6	70	0	180	85	5.0		B.P. returned to control level in 4 days; died 78 days later. Adrenal cortex hypertrophied at autopsy
					30	230	130			
					65	220	125	5.0	Spas. mus.	
W76, ♀	14	Normotensive	0.7	70	0	160	85	5.5		B.P. elevation persisted for 62 days
					15	130	60		Weak	
					28	135	80	3.4	Weak	
					56	155	100	3.4	Active	
Y1, ♂	11	R.N.; temporary L.C. (1½ hr.). Spontaneous hypertensive	0.8	45	0	180	100			B.P. fell to normal in 24 days
					36	200	125			
X34, ♂	9	R.N. Normotensive	1.0	72	0	160	75	4.7		B.P. remained at high levels. Died 40 days later with hemorrhages in mesentery and lung
					25	190	110	3.7		
					70	210	115			
X8, ♂	23	R.C., L.C. Hypertensive	0.4	72	0	180	110	8.2[2]		
					26	175	115		Spas. mus.	
					65	185	120	3.1		
X61, ♂	19	R.C., L.C. Hypertensive	0.5	19	0	200	115			Died on 19th day of injection, with acute left heart failure and acute pulmonary edema
					7	240	135			
					15	225	125			
X54, ♀	14	R.N., L.C. Hypertensive	0.7	21	0	200	100	4.4		Died on 21st day of injection, with acute left heart failure and acute pulmonary edema
					12	220	130			
					18	210	150			
X83, ♂	14	R.N., L.C. Hypertensive	0.7	70	0	180	105	3.9		B.P. returned to normal in 14 days
		..			50	195	120			
					60	200	120	4.2		
Y103, ♂	14	R.C., L.C. Hypertensive	0.7	45	0	200	100			B.P. persisted at elevated level for 40 days
					15	215	115			
Y41, ♂	11	R.C., L.C. Hypertensive	0.9	66	0	200	105	5.4		Rise in B.P. maintained for 40 days
					15	175	115			
					22	300	180	3.0		

[1] R.N.=right nephrectomy; R.C.=right renal artery constricted; L.C.=left renal artery constricted.
[2] Sample hemolyzed.
[3] Spas. mus.=spastic muscles.

did serum calcium, sodium, glucose, phosphates or chlorides show any significant alterations. However, serum potassium was reduced as much as 50 per cent. This change in serum potassium could not be correlated with the changes in blood pressure.

In one animal, a normotensive, (X49) under desoxycorticosterone acetate administration, hypertrophy of the adrenal cortex with almost complete obliteration of the medulla was observed at autopsy. In two cases, hypertensives (X61, X54), acute pulmonary edema was observed. In one case, a normotensive (X34), extensive

hemorrhages were found in the viscera. Otherwise no gross pathologic abnormalities were observable.

Several of the dogs showed spasticity of the muscles or muscular weakness during desoxycorticosterone acetate administration. This has been observed previously (1).

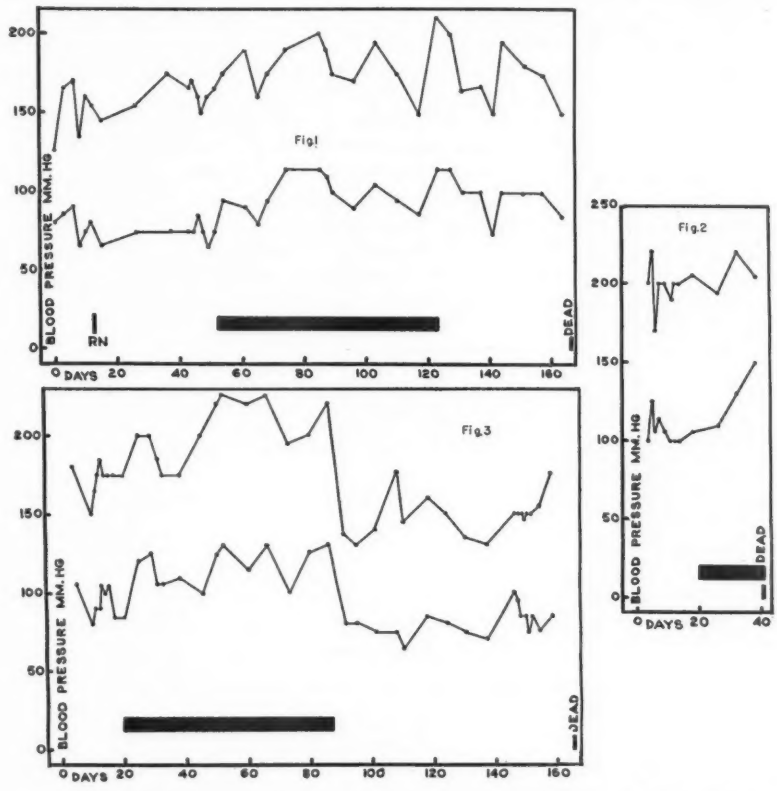


Fig. 1. Effect of desoxycorticosterone acetate on the blood pressure of a normotensive uninephrectomized dog (×34). *RN* shows time of right nephrectomy. Solid horizontal block shows period of desoxycorticosterone acetate administration, 20 mg./day.

Fig. 2. Effect of desoxycorticosterone acetate on the blood pressure of a Goldblatthypertensive dog (×54). Conventions as in figure 1.

Fig. 3. Effect of desoxycorticosterone acetate on the blood pressure of a spontaneously hypertensive dog (×49). Conventions as in figure 1.

DISCUSSION

There was no significant difference in the nature of the response in the normotensive, spontaneously hypertensive and Goldblatt-hypertensive animals as regards the degree of blood pressure change or the direction of the blood chemistry changes. In two of the Goldblatt-hypertensive dogs the blood pressure rise superimposed on a preexisting hypertension was sufficient to precipitate acute heart failure and pulmonary edema. The development of this edema might have been due in part to the loss of serum potassium with the relative retention of sodium chloride.

The hypertensive effect of large doses of desoxycorticosterone acetate in both normal and previously hypertensive dogs suggests that this drug acts in a similar manner in both types of animals. This action may be due to a direct effect on the smooth muscle of the arterioles, or on the metabolism of the kidney in effecting the production of the chemical mediator of hypertension. On the other hand, the electrolyte metabolism changes due to administration of desoxycorticosterone acetate may be instrumental in the elevation of blood pressure. In this regard it has been demonstrated that in the treatment of Addison's disease with desoxycorticosterone acetate, an excess of NaCl intake will augment the increase in blood pressure (5).

There is evidence in the work of Goldblatt (9) and others (10, 11) that the maintenance of experimental renal hypertension is dependent upon the presence of the adrenal glands. Furthermore, several workers (12, 13, 14) have suggested that the adrenals are essential for the potentiation of the action of renin. However it would appear that the actual presence of the adrenals is not necessary for the production of acute nephrogenic hypertension (15, 16). In this regard it is interesting to note that a case of adrenal insufficiency with hypertension has recently been reported (17).

In conclusion, our work indicates that the action of desoxycorticosterone acetate is similar in Goldblatt-hypertensive, spontaneously hypertensive and normotensive animals.

SUMMARY

Daily injections of 20 mg. of desoxycorticosterone acetate to normotensive, spontaneously hypertensive and Goldblatt-hypertensive dogs led to elevations of blood pressure in 9 of 12 animals.

In two of the previously hypertensive dogs, acute pulmonary edema developed and death ensued.

The blood pressure changes after cessation of injections of desoxycorticosterone acetate led a variable course; in some the elevated pressure remained for a considerable period, in others it fell rapidly or slowly to the control pressure levels.

The fall in serum potassium attendant upon administration of desoxycorticosterone acetate was not related to the blood pressure elevation.

We wish to express our gratitude to Dr. L. N. Katz for his guidance in this study.

REFERENCES

1. Ferrebee, J. W., C. Ragan, D. W. Atchley and R. F. Loeb: *J. Am. Med. Assoc.* 113: 1725. 1939.
2. McGavack, H.: *J. Clinical Endocrinology* 1: 68. 1941.
3. Thorne, G. W.: *J. Clin. Investigation* 1: 76. 1941.
4. Soffer, L. J., F. L. Engel and B. S. Oppenheimer: *J. Am. Med. Assoc.* 115: 1860. 1940.
5. Swingle, W. W., W. M. Parkins and J. W. Remington: *Am. J. Physiol.* 134: 503. 1941.
6. Grollman, A., T. R. Harrison and J. R. Williams: *J. Pharmacol. & Exper. Therap.* 69: 149. 1940.
7. Kuhlmann, F., C. Ragan, J. W. Ferrebee, D. W. Atchley and R. F. Loeb: *Science* 90: 496. 1939.
8. Hamilton, W. F., J. Brewer and I. Brotman: *Am. J. Physiol.* 107: 427. 1934.
9. Goldblatt, H.: *Ann. Surg.* 106: 826. 1937.
10. Page, I. H.: *Am. J. Physiol.* 122: 352. 1938.
11. Collins, D. A., and E. H. Wood: *Am. J. Physiol.* 123: 224. 1938.
12. Williams, J. R., J. T. Diaz, J. C. Burch and T. R. Harrison: *Am. J. M. Sc.* 198: 212. 1939.
13. Friedman, B., E. Somkin and E. T. Oppenheimer: *Am. J. Physiol.* 128: 481. 1940.
14. Fasciolo, J. C.: Rev. Soc. argent. de biol. 14: 25. 1938.
15. Rogoff, J. M., E. N. Nizon and G. N. Stewart: *Proc. Soc. Exper. Biol. & Med.* 41: 57. 1939.
16. Enger, R., F. Linder and H. Sarre: Ztschr. f. d. ges. exper. Med. 104: 10. 1939.
17. Hampton, H. P., and E. J. Kepler: *Am. J. M. Sc.* 202: 264. 1941.

THE EFFECT OF EPINEPHRINE UPON SEMEN PRODUCTION IN THE DOMESTIC FOWL

NORMAN C. WHEELER, GLENN L. SEARCY
AND FREDERICK N. ANDREWS[1]

From the Agricultural Experiment Station, Purdue University
LAFAYETTE, INDIANA

DURING THE PAST TWO DECADES evidence of a relationship between the adrenal medulla and the gonads has been accumulating. Chidester (1) reported that when fowls were fed desiccated whole adrenal gland for 10 days body weight decreased. Eaton et al. (2) observed that when chicks were fed 0.5 grains of suprarenal medulla daily from the 12th to 89th day of life they weighed 53.75 grams less than the controls and that the testes of the controls were 37 per cent heavier than the gonads of the medulla-fed males.

Riddle and Minoura (3) transplanted whole adrenal glands into ring doves and found that the testes of birds receiving the transplants were smaller than those of the controls but that the ovaries of transplanted females were larger than the ovaries of the controls. Although the females receiving transplants layed at an early age all of the eggs were infertile, indicating, perhaps, that the males receiving transplants might have been producing no functional spermatozoa.

Eguchi (4) observed that repeated subcutaneous injections of epinephrine caused testicular atrophy in the young and mature rabbit and the adult guinea pig. He described this phenomenon as 'adrenalin poisoning,' stating that it was characterized by marked degeneration of the interstitial cells and that spermatogenesis was affected in varying degrees depending upon the dosage of epinephrine.

Perry (5) reported that 15 to 20 one-half minim doses of epinephrine (1:1,000) daily, injected into the pectoral muscles of normally reproducing male and female English sparrows or into those brought into the breeding condition by artificial light or gonadotropic hormones, caused a regression of the gonads, accessories and secondary sex characteristics to the juvenile condition. Perry (6) extended this study to the rat and found that experimental hyperadrenalism resulted in the atrophy of the seminal vesicles, prostate, epididymis and vas deferens, and in some instances caused marked degeneration of the germinal epithelium and the complete suppression of spermatogenesis.

MATERIALS AND METHODS

These experiments were conducted between June 23 and Sept. 26, 1941. Eight mature barred Plymouth Rock males were selected at random from a large group of apparently normal cocks. Semen was collected three times weekly by the massage method of Burrows and Quinn (7) and measured to the nearest 0.01 cc. The concentra-

Received for publication December 4, 1941.

[1] We are indebted to Dr. K. K. Chen, Director of Pharmacologic Research of the Lilly Research Laboratories, Indianapolis, Indiana, for his interest in this study and for the epinephrine used in these experiments, to Drs. L. P. Doyle and A. L. Delez of the Veterinary Science Department, Purdue, for assistance in the interpretation of the testis sections, and to Dr. W. R. Breneman, Department of Zoology, Indiana University, for criticism of the manuscript.

tion of spermatozoa per cubic millimeter was determined by the use of the hemacytometer.

The semen was stored at approximately 6°C. and the motility of the spermatozoa in each sample was observed daily until all cells had lost motility. The weekly feed consumption was determined for each bird and the body weights were recorded at seven-day intervals.

The experiment was divided into three phases, a 14-day pre-injection period, a 30-day injection period, and a 49-day post-injection period.

The epinephrine solution was made up of 0.195 gm. of epinephrine dissolved in 12 cc. of N/2 HCl and diluted with distilled water to make 195 cc. This solution was made up weekly, saturated with CO_2 and stored in a dark bottle at 5 to 6°C. Injections were made into the pectoral muscles and care was taken to alternate the site of injection. Four males (*Group I*) received the approximate daily dose which Perry used in the sparrow, 1.21 cc. of 1:1,000 epinephrine per kg. of body weight and four cocks (*Group II*) were given 0.6 cc. per kg. of body weight.

The males were autopsied at the close of the experiment and testis blocks were immediately fixed in Bouin's fluid and prepared for microscopic study.

RESULTS

The volume of semen dropped sharply in both groups following the injection of epinephrine and had decreased approximately 48 per cent at the end of the injection

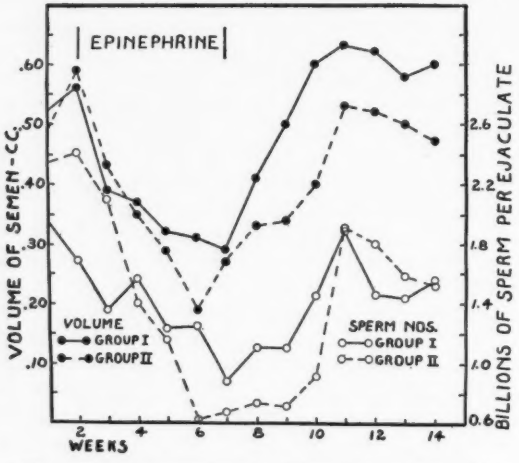


Fig. 1. Effect of epinephrine upon semen volume and concentration of spermatozoa.

period (fig. 1). Following the cessation of injections there was an immediate increase in semen volume and approximate pre-injection levels were reached in 4 weeks. Since the males in *Group II* responded similarly to those in *Group I* it would seem that 0.6 cc. of 1:1,000 epinephrine was as effective as the heavier dosages.

Although the determination of the concentration of spermatozoa by the hemacytometer is subject to an error of approximately 10 per cent (8) certain trends were evident. The number of sperm per unit volume of semen was not markedly affected until the last week of epinephrine injections but the total number of spermatozoa per

ejaculate followed the same trend as semen volume (fig. 1). At the end of the injection period the total output of sperm had decreased approximately 50 per cent in *Group I* and 70 per cent in *Group II*. The concentration of spermatozoa per unit volume did not begin to increase until the 4th week following the cessation of injections and had not reached pre-injection levels at the termination of the experiment. The total

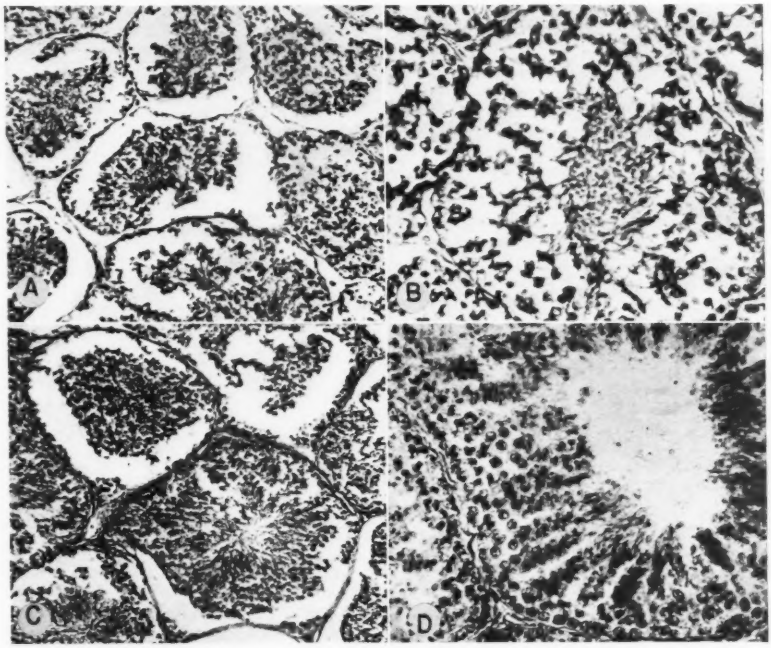


Fig. 2. EFFECT OF EPINEPHRINE UPON TESTIS. *A. Male 7007, Group I.* The seminiferous tubules are atrophied and dissociated. ×80. *B. Male 7007, Group I.* All germinal elements including the spermatogonia are dissociated. Cytoplasmic and nuclear damage are evident. ×170.

C. Male 7011, Group II. Same type of damage that occurred in males of *Group I* is apparent although to a lesser degree. ×80. *D. Male 7023, Group II.* Relatively normal tubule, not typical of the entire testis, but showing the characteristic architecture. Adjacent tubules showed definite dissociation. ×170.

number of spermatozoa per ejaculate followed the same general trend as semen volume but full recovery had not been reached at the time of autopsy.

Observations on the maintenance of motility of sperm stored at 5 to 6°C. showed that prior to the injection of epinephrine, motility was maintained for approximately 10 days but decreased to 5 days in both groups after 3 weeks of injection. Spermatozoa obtained approximately 3 weeks after treatment had been stopped, retained motility for 9 days.

Although the males were autopsied 7 weeks after epinephrine injections had been terminated the testis sections showed varying degrees of damage. The seminiferous tubules of the males which received the larger doses of epinephrine were, without exception, shrunken and the cells were markedly dissociated. The typical architecture of the germinal elements was lacking; the spermatogonia were, in some cases, dissociated from the normal basal position; primary and secondary spermatocytes, sper-

matids and spermatozoa, although present, were apparently still being produced at decreased rates and their relationships, one to another were changed from the normal. Individual cells of all types frequently showed degenerative cytoplasmic changes and in some instances nuclear damage was also apparent (fig. 2). Three of the males in *Group II* showed testicular damage of the type previously described and only one male possessed relatively normal gonads (fig. 2).

There was an immediate decrease in food consumption following epinephrine treatment (fig. 3), and at the end of 4 weeks food intake had decreased approximately

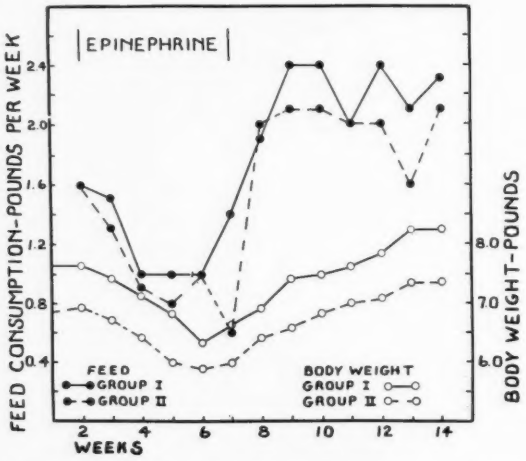


Fig. 3. Effect of epinephrine upon feed consumption and body weight.

38 per cent. Following the cessation of injections an immediate rise in food consumption occurred and the males consumed approximately 30 per cent more food per week than at the beginning of the experiment.

As would be expected, decreased food intake was paralleled by a distinct loss in body weight and at the end of the injection period the males in *Groups I* and *II* were 17 and 14 per cent lighter respectively than at the beginning of the experiment. Following the termination of injections there was a rapid increase in body weight and at the time of autopsy the birds were heavier that at the beginning of the experiment (fig. 3). An examination of the males at the time of autopsy showed that they were plump, firmly fleshed, and there was no evidence of edema.

DISCUSSION

The results of this study tend to bear out the findings of Perry (5, 6) and others that large doses of epinephrine interfere with gonad function but the specific mechanisms responsible for this apparent epinephrine-gonad antagonism remain incompletely explained.

Although epinephrine is usually considered to be a highly specific stimulant of the entire sympathetic system, particularly the vasoconstrictor mechanism of the systemic blood vessels and the accelerator mechanism of the heart, many other actions have been described. The injection of large doses of epinephrine results in marked hyperglycemia and glycosuria, and increased body metabolism. Inhibition of the bladder, stomach, gall-bladder, and pancreas have been described and fatal doses in animals

have been accompanied by diarrhea, hematuria, marked fall of body temperature and complete prostration (10).

The sensitivity of the testis to many specific substances and to unbalanced physiological conditions in general has often been demonstrated and caution must be exercised in drawing conclusions as to the mode of action of epinephrine in testis inhibition. Arsenic, alcohol, iodine, opium and other substances have often been reported to cause testicular degeneration. Titus and Burrows (9) found that a steady decrease in semen volume followed the feeding of desiccated thyroid to the domestic cock and that even the addition of 0.5 per cent of cold-pressed wheat germ oil (which is high in vitamin E and might be expected to be beneficial) to the ration of the fowl caused a marked decrease in semen production.

Perry (5) reports that gonad suppression in the English sparrow following epinephrine injection is probably the result of decreased secretion, release or activity of the anterior pituitary gonadotropins. In the rat (6) there was considerable variation in the testicular damage which followed the injection of epinephrine and the interruption of spermatogenesis was most marked in rats which were injected during the summer months. Perry (6) concludes that the mode of action in the rat differs from that in the sparrow and suggests that the calorigenic action of epinephrine in conjunction with the high environmental temperatures of the summer months may have been partially responsible for the depression of spermatogenesis.

Although, to our knowledge, the effects of epinephrine upon semen production itself have not previously been reported, the results are sufficiently striking to warrant the conclusion that epinephrine does markedly affect semen production and spermatogenesis. However, the various explanations which have thus far been proposed as to the mechanism of the epinephrine-gonad antagonism must be substantiated by further and more critical research. Although decreased pituitary activity following epinephrine injections could easily account for the gonadal changes which have been described it would appear necessary to study the gonadotropic hormone content of the hypophysis before the rôle of the pituitary can be understood.

Since the testis is apparently responsive to unbalanced physiological states resulting from many causes, the general body effects of epinephrine should not be overlooked. The 38 per cent decrease in food intake which was observed after 4 weeks of epinephrine injections and the 14 to 17 per cent decrease in body weight during the injection period are indications that the entire organism was being affected. Although other workers have not reported data on food consumption, the findings of Chidester (1) that the feeding of whole adrenal glands for 10 days caused a loss of body weight in the fowl, of Eaton et al. (2) that chicks fed suprarenal medulla grew at a decreased rate, and of Perry (6) that some groups of male rats lost as much as 30 per cent of body weight following epinephrine injections would suggest that decreased food intake may have been a factor in the observed results.

It is clear that in the fowl epinephrine injections are followed by decreased semen volume, decreased concentration and total numbers of spermatozoa, decreased viability of spermatozoa, dissociation of the seminiferous tubules, and decreased food consumption and body weight. The mechanisms responsible for these observed changes are not understood and as to whether epinephrine acts directly upon the testis, indirectly through the pituitary or indirectly by affecting all physiological processes in general remains to be shown.

SUMMARY

1. The intramuscular injection of epinephrine was followed by an almost immediate decrease in semen volume with an approximate 48 per cent reduction at the

end of a 30-day-injection period. Volume rose to pre-injection levels following the cessation of treatment.

2. The concentration of spermatozoa decreased after 3 weeks of injections and had not risen to pre-injection levels at the close of the experiment.

3. The total number of sperm per ejaculate decreased 50 to 70 per cent during the injection period.

4. The survival time of spermatozoa was decreased approximately 50 per cent by epinephrine.

5. Marked dissociation of the cells of the seminiferous tubules was present 7 weeks after the cessation of injections.

6. Feed consumption was decreased approximately 38 per cent during treatment.

7. Body weight decreased during the injection period but final weights were greater than at the beginning of the experiment.

REFERENCES

1. Chidester, F. E.: *Science* 36: 641. 1912.
2. Eaton, A. G., W. M. Insko, G. P. Thompson and F. E. Chidester: *Am. J. Physiol.* 88: 187. 1929.
3. Riddle, O., and T. Minoura: *Proc. Soc. Exper. Biol. & Med.* 20: 456. 1923.
4. Eguchi, K.: *Jap. Jikkew Igaku Zasshi.* 11: 1129. 1927.
5. Perry, J. C.: *Anat. Rec.* 79: 57. 1941.
6. Perry, J. C.: *Endocrinology* 29: 592. 1941.
7. Burrows, W. H., and J. P. Quinn: *Poul. Sci.* 15: 19. 1937.
8. Weisman, A. I.: Spermatozoa and Sterility. P. B. Hoeber Inc., New York, 1941. P. 56.
9. Titis, H. W., and W. H. Burrows: *Poul. Sci.* 19: 295. 1940.
10. Sollmann, T.: A Manual of Pharmacology. W. B. Saunders Co., Philadelphia, 1936. P. 405.

RELATION OF GLAND WEIGHTS TO GROWTH AND AGING PROCESSES IN RATS EXPOSED TO CERTAIN ENVIRONMENTAL CONDITIONS[1]

L. P. HERRINGTON AND JEAN H. NELBACH

From the John B. Pierce Laboratory of Hygiene

NEW HAVEN, CONNECTICUT

IN TWO EARLIER PAPERS (1, 2) the effect of various environmental conditions upon the growth of rats and upon the tensility of the aorta has been reported. These studies showed that the tensility factor (measured by the distention of an aortal ring under gradually increasing stress) varied with age, the aortae of older animals becoming progressively less resistant to a given tension.

Under certain experimental conditions, the normal curve of tensility was profoundly modified. Rats kept at a low temperature (65°F.) had aortae with high tensility values, resembling those of much younger animals. Rats kept at a high temperature (95°F.), rats receiving thyroid extract, and rats exposed to long term nervous disturbance (intermittent bell ringing, air blasts, and cage vibration once each hour) all had aortae with low tensility resembling that of older animals. The influence of these various treatments on general growth has been reported in connection with evidence that the tensility differences associated with the various treatments are not merely the reflection of differences in growth.

In the present paper are presented: *a*), complete data on weight and length at 120 and 210 days of age for the experimental groups of animals studied in 1939 and 1940; *b*), new data on the weight of the heart, liver, pituitary, adrenals, testes, prostate and seminal vesicles; and *c*), a report on the histologic studies of the aortic sections. The analyses are based on the comparison of differences found in 1939 between the animals autopsied at 120 days and those at 210 days, because the principal significance is dependent upon continuity in the experiment. In 1940, three groups of animals were autopsied at 120 days as controls for tensility measurements. The results of these studies are summarized in the tables, but the organ weights for these groups are not considered in the discussion since the conspicuous effects appear principally at 210 days.

The data on body growth are summarized in figure 1 for the 1939 and 1940 experiments. The mid-line indicates the 120-day point at which 20 animals from each group were autopsied in 1939. The 120-day growth curves in 1940 represent only 20 animals. These results were obtained as a final check on the wide tensility differences noted between *65°F. animals* and *disturbed animals*.

It will be noted that there was a tendency for the groups of rats stimulated by thyroid feeding and disturbance to mature earlier and to reach higher levels of body weight than groups maintained at 65° or 95°F. Several minor features deserve comment. In the 1939 data, reduction of cage population from 40 to 20 animals at 120 days resulted in an increased growth rate, a finding further confirmed by the initially greater growth of the 1940 population of 20 animals. Also, it appears probable that a temperature of 95°F. is relatively favorable for growth in the period from 60 to 100 days and is unfavorable therafter. The reverse is true of a temperature of 65°F. at

Received for publication June 24, 1941.

[1] Contribution No. 34.

which growth is distinctly greater. The effect of cage 'crowding,' and the change in optimal temperature for growth at various stages of maturity, are probably related respectively to food competition and the effect of increasing body size on temperature susceptibility.

ORGAN WEIGHTS IN RELATION TO BODY WEIGHT

Previous work has indicated a decrease in aortic tensile resistance correlated with age and with increasing body weight. The weight effect is most conspicuous in the

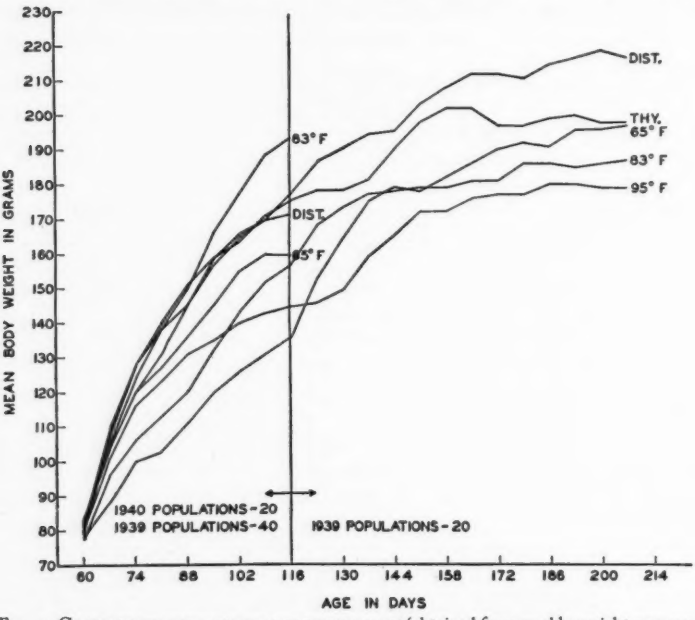


FIG. 1. GRAPH OF THE 1939 AND 1940 GROWTH CURVES (obtained from weekly weight measurements) after smoothing with a moving average of three. Double arrow indicates age at which 20 animals from each of the 1939 groups were autopsied.

first 210 days (2). It was established, however, that at standard age or weight the various treatments of heat, cold, thyroid and disturbance produce definite effects on aortic tensility independent of age or weight. If we accept the decrease in tensile resistance with age as an unfavorable effect, it is clear that high temperatures, thyroid feeding, and disturbance are unfavorable treatments, while a cold temperature is definitely favorable. The independence of such favorable or unfavorable effects from general growth or age is emphasized by the fact that the *disturbed* and 95° *temperature groups*, which had the two lowest values for tensile resistance at the age of 210 days were, respectively, the heaviest and the lightest animals in body weight.

In table 1 are given the body weights, body lengths and weight-length ratios for all groups of rats at both 120 and 210 days of age. Table 1 shows that at both 120 and 210 days the animals in the thyroid group and the disturbed group had the greatest body lengths and that those of the 95° temperature group were among the shortest. Consequently it was not surprising to find that weight, length, and the weight-length ratios all have a similar relation to tensile performance. Although treatment effects on tensile resistance of the aorta appeared to be independent of general growth,

TABLE 1. GENERAL GROWTH DATA ON GROUPS OF 20 RATS UNDER VARIOUS EXPERIMENTAL CONDITIONS

Expt. No.	Age in Days	Special Treatment	Av. Final Wt., gm.	Initial Wt., gm.	Av. Gain, gm.	Av. Gain in Wt. 120–210 Days	Weight/ Length Ratio	Order of Weight/ Length Ratio	Av. Length, cm.	Av. Gain in Length 120–210 Days, cm.
					60–120 days			*120 days*	*120 days*	
9	120	65° F.	145	72	73		8.80	3	16.7	
10	120	83° F.	155	79	76		8.60	5	18.1	
11	120	95° F.	150	80	70		8.76	4	17.0	
12	120	Thyroid	188	79	109		9.66	1	20.0	
13	120	Disturbed	176	81	95		8.88	2	19.8	
19	120	65° F.	168	84	84		8.90	2	18.8	
20	120	83° F.	203	84	119		10.12	1	20.0	
21	120	Disturbed	170	83	87		8.85	3	19.1	
					60–210 days			*210 days*	*210 days*	
14	210	65° F.	217	72	145	72	10.29	2	20.1	3.4
15	210	83° F.	204	79	125	49	10.24	3	19.9	1.8
16	210	95° F.	184	80	104	34	9.48	5	19.4	2.4
17	210	Thyroid	204	79	125	16	9.82	4	20.8	0.8
18	210	Disturbed	223	81	142	47	10.90	1	20.4	0.6

whether measured by body weight, body length, or weight-length ratio, it seemed possible that differences in the growth of various body organs in relation to body weight might be associated with the effects of treatment.

In table 2 are given the absolute organ weights and weights relative to body weights for the liver, heart, testes, adrenals, prostate and seminal vesicles, and pituitaries of all groups. At 120 days of age the outstanding deviations in organ weight per gram of body weight involved principally the liver and testes. Cold temperatures and thyroid feeding were associated with large livers at 120 days of age. The normal temperature of 83°F. or cold (65°F.) seemed to be associated with greater testicular development. The liver weight did not appear to be related to favorable or unfavorable effects on aortic tensility. The testicular weights indicate an association of high aortic tensility and greater testicular development.

It can be concluded that no simple pattern in organ-weight relationship distinguishes the groups of animals with high and low tensile resistance at 120 days of age.

TABLE 2. AVERAGES OF ABSOLUTE AND RELATIVE[1] ORGAN AND GLAND WEIGHTS FOLLOWING EXPERIMENTAL TREATMENT

Exp. No.[2]	9	10	11	12	13	19	20	21	14	15	16	17	18
Age in days	120	120	120	120	120	120	120	120	210	210	210	210	210
Exper. Treatment	65°F.	83°F.	95°F.	Thy.	Dist.	65°F.	83°F.	Dist.	65°F.	83°F.	95°F.	Thy.	Dist.
Final Body Wt. (gm.)	145	155	150	188	176	168	203	170	217	204	184	204	223
Wt. Liver (gm.)	7.79	7.52	6.55	10.45	8.68	8.66	9.52	8.11	9.98	9.30	8.45	9.46	10.27
Rel. Wt. Liver (mg.)	52.6	48.4	43.8	55.6	49.2	51.5	46.9	47.6	46.0	45.7	45.8	46.3	46.1
Wt. Heart (gm.)	0.48	0.51	0.46	0.64	0.58	0.59	0.60	0.56	0.75	0.72	0.65	0.92	0.79
Rel. Wt. Heart (mg.)	3.3	3.3	3.1	3.4	3.3	2.9	3.3	3.5	3.5	3.5	3.5	4.5	3.6
Wt. Testes[3] (gm.)	1.96	2.12	1.60	2.10	2.08	2.17	2.38	2.10	2.19	2.26	1.77	1.90	2.02
Rel. Wt. Testes (mg.)	13.2	13.6	10.7	11.2	11.8	12.9	11.7	12.4	10.1	11.1	9.6	9.3	9.1
Wt. Adrenals[4] (mg.)	31.2	24.7	22.2	27.6	24.1	30.3	30.7	28.2	24.5	23.3	16.8	29.0	31.9
Rel. Wt. Adrenals (mg.)	0.14	0.16	0.15	0.15	0.14	0.18	0.15	0.16	0.11	0.11	0.09	0.14	0.14
Wt. Prostate[5] (gm.)	0.87	1.06	1.01	1.08	1.15	1.19	1.40	0.98	2.19	1.64	1.86	1.00	1.49
Rel. Wt. Prostate (mg.)	5.9	6.8	6.8	5.7	6.5	7.1	6.9	5.8	10.1	8.6	10.1	4.9	6.7
Wt. Pituitary (mg.)	5.7	6.7	6.5	7.3	6.8	7.5	7.4	6.5	8.4	5.7	7.0	7.8	4.2
Rel. Wt. Pituitary (mg.)	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.04	0.04	0.02

[1] Relative weight is weight of each organ or gland per gram of body weight.
[2] Experiments 9–18 were completed in 1939. Experiments 19–21 were completed in 1940.
[3] Both testes were weighed together.
[4] Adrenals were weighed together.
[5] Prostate and seminal vesicles were weighed together.

At 210 days of age more striking differences were apparent, particularly the great development of the heart in the *thyroid group*, and a maintenance of the association between high testicular weights and high tensile resistance.

A consideration of the effects of various treatments on body, gland and organ weights involves the simultaneous consideration of many interrelated variables. We can probably expect greatest insight from the discovery of pattern effects. Such comparisons are most easily made graphically and it is desirable to express all results in terms of an appropriate standard. Donaldson's (3) data on organ weights in relation to body weight are not ideal, since both the growth curve and the organ constitution of the Wistar strain of rats differ considerably from similar data for our colony. We have expressed body and organ weights in terms of a percentage of the corresponding values obtained from our control animals maintained at 83°F. By this procedure factors of diet, age and strain are directly controlled.

PATTERN EFFECTS OF ORGAN WEIGHTS AS RELATED TO TREATMENT

In table 3 are presented the values at 210 days of age for body, liver, heart, testes, adrenals, prostate and seminal vesicles, and pituitary weights for the 65° *temperature*,

TABLE 3. BODY, ORGAN, AND GLAND WEIGHTS OF ALL 210-DAY-OLD GROUPS IN 1939 IN PERCENTAGE OF THE 83° F. GROUP

	65° F.	95° F.	Thyroid	Disturbed
	%	%	%	%
Body weight	106	90	100	109
Liver	107	91	102	110
Heart	104	90	127	110
Testes	97	78	84	89
Adrenals	105	72	124	137
Prostate, Sem. Ves.	134	113	61	91
Pituitary	148	123	138	74

95° *temperature*, *thyroid* and *disturbed groups* as a percentage of the corresponding weights for the 83° *temperature controls*. In figure 2 these percentages have been plotted. The order has been arbitrarily determined so that body, liver and heart weights are plotted in succession on the abscissa, followed by the weights of the four endocrine glands. The mid-line represents the 100 per cent value which, in each case, is taken as that of the corresponding item for the 83° *temperature controls*. In the left portion of the graph the results for the 65° *temperature* and 95° *temperature groups* are together, on the right are the results for the *thyroid group* and the *disturbed groups*. It is apparent that there are two patterns in these weights that are typical, respectively, of temperature effects (65° temperature and 95 temperature) and of stimulative effects (thyroid and disturbance). The similarity within each pair of curves and the contrast between the right and left hand plots are striking. If these curves are plotted with a body-weight correction, the similar features are practically superimposed. Hence, we may accept these relations as valid on either basis.

A comparison of body and organ-weight profiles of the temperature groups shows a close correspondence. It is probably justified to say that a shift in habitat temperature above or below thermal neutrality at 83°F. produces a typical pattern, with the *cold temperature group* attaining a higher general level of growth. Assuming a divergence of ± 10 per cent to be within a normal physiological range, it is obvious the body, liver and heart weights were not affected in development by either a high or low environmental temperature. The differences in general level of growth can be accounted for in terms of Kleiber and Dougherty's (4) conception of optimal temperatures and

food energy available for growth. The outstanding deviations appear to be the result of temperature effects on the balance of the endocrine system as manifested by the small testicular and adrenal weights in the 95° *temperature group* and large prostatic and pituitary weights in both the 95° *temperature group* and the 65° *temperature group* as compared with the 83° *temperature group*. The question of temperature influence on endocrine function, and of the relation of such influences to the apparent aortic deficiencies of the 95° *temperature group* is of obvious interest.

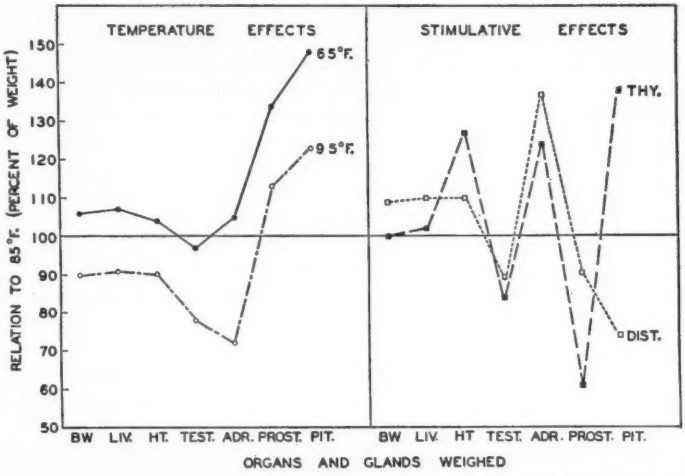


FIG. 2. GRAPH OF THE PATTERN EFFECT of average organ and gland weights expressed in percentages of the corresponding weights for the 83° temperature group. All groups represented were 210 days of age.

The relationship is clearest in connection with adrenal function. Alpern (5) has presented evidence for the rôle of the sympathetic nervous system in the control of tissue metabolism, and it is generally recognized that the peripheral heat-regulating mechanism is under sympathetic control (6). It is, therefore, not surprising to find that the adrenals are comparatively larger in the 65° *temperature group* and smaller in the 95° *temperature group*, since exposure to cold is known to stimulate the sympathetic nervous system and the related endocrines.

Moore (7) in studies of experimental cryptorchidism related testicular function to body temperature, and, therefore, to environmental temperature. Studies previously published from this laboratory (8) indicate that the average rectal temperature of the rat in an environment of 95°F. is approximately 103°. Since the dissipation of body heat from the internal parts to the surface and to the air requires a continuous gradient, an environmental temperature of 95° necessitates testicular and scrotal temperatures of the order of 98° or 99°F. These values are approximately the same as the normal body temperature in environments of 70° to 80°F. The testicular retardation found in the 95° *temperature group* may be clearly related to abnormally high scrotal temperatures. It is perhaps justified to conclude that the 95° *temperature group* experiences a type of partial physiological castration except that the increased weights of the prostate and seminal vesicles are not consistent with the results of experimental castration. It is conceivable, however, that the prostatic hypertrophy and testicular atrophy in the 95° *temperature group* are similar to the benign prostatic hypertrophy

associated with diminution of testicular function with age. It is of considerable interest that both the endocrine pattern and the aortic properties of the 95° *temperature group* are distorted in a manner usually associated with an effect of age.

Hill (9) reported work on the effect of temperature on ovarian function which emphasizes the negative association of high temperatures and androgenic function and supplements the established effects of temperature on spermatogenesis. Temperature is obviously important in determining the balance of androgenic-estrogenic function in the ovary. If the rat testis possesses a similar dual function as has been shown for the testis of the stallion, the prostatic hypertrophy in the 95° *temperature group* could conceivably be due to an actual or relative increase in estrogenic output (10). The weight of the testes in the 65° *temperature group*, although lower than that for the general level of growth, is not considered abnormal.

The enlarged pituitary in the 95° *temperature group* may be an accompanying reaction from hypoplastic testicles which elicit increased output of the pituitary gonadotropic hormone, or it may be a hyperplasia elicited by the increase in estrogenic output as suggested. In a sense, the overgrowth of the pituitary in the 95° *temperature group* was due to the high temperature which primarily affected the development of the testes, resulting in hyperpituitary eunuchoidism. The growth curves for all of the groups in 1939 showed that the effect of a 95° temperature in the cage was to produce a type of animal whose length in proportion to its weight was great at all times. The size of the pituitary is generally larger in tall individuals and is not correlated with body weight. A length factor is also related to the large pituitary weight found in the animals of the 65° *temperature group*, since of the three temperature groups they grew more in length between 120 and 210 days than either those of the 83° *temperature group* or of the 95° *temperature group*.

In the right section of figure 1 similar curves have been plotted for the thyroid-fed and chronic-disturbance groups. A strong similarity in the endocrine weight patterns following stimulative procedures influencing sympathetic and thyroid-adrenal action is apparent. These results should be considered in relation to Selye's work on the alarm reaction (11). Our experiments did not involve the extremes of stimulation characteristic of Selye's work, but some relationship is evident. In both the *thyroid group* and the *disturbed group* the adrenal weights are greater and testicular weights are less, which agrees with Selye's findings. The reactions specific for the *thyroid group* involve an increase in heart and pituitary weights similar to that seen in the animals of the 65° and 95° *temperature group*. The *disturbed group* differ from the common pattern chiefly in the markedly reduced pituitary weight, and a less conspicuous reduction in prostatic weight. The important differences between the *thyroid group* and the *disturbed group* are probably associated with the use of thyroid feeding in one group. The pattern of increased pituitary and adrenal weight in the *thyroid group* and the decreased weight of the testes, seminal vesicles and prostate is also very similar to that noted by many investigators as the result of the injection of estrogenic substances into normal adult rats (12). Although the relation of the thyroid to the gonads is not completely understood, some of the existing evidence on thyroid feeding in male fowls suggests an estrogenic rather than an androgenic effect, since male birds fed small doses of thyroid do not regenerate characteristic male plumage after plucking (10). Clinical sources also often associate hyperthyroidism with a vaguely defined 'hypogonadal' state in both sexes.

The most characteristic difference in the patterns of the *disturbed* and the *thyroid groups* is the small pituitary weights of the *disturbed group*. This group has the largest adrenals and it is conceivable that these are initially involved, since the treatment was primarily one of excitement. The decreased pituitary weight, increased adrenal

weight and decreased testis weight agree roughly with the results noted by Vidgoff and Vehrs (13, 14) as a result of testicular injections in rats. If there is any inference to be drawn it seems possible that it might be related to an androgenic effect of adrenal cortical origin.

The interpretations suggested above are frankly speculative and need serious investigation with entirely different techniques. Our interest was centered principally on aortic tensility results, and it was surprising to find that later consideration of the more or less routine endocrine weight determinations was so suggestive. The consistency with which all of the procedures which produced age-mimetic effects on aortic tensility, such as increased temperature, thyroid feeding and chronic disturbance, were associated with retarded testicular development is noteworthy. It is unlikely that three distinct techniques, producing a similar gonadal effect, would be associated with similar age-mimetic effects on the aorta purely as a matter of chance. The influence of the gonadal principles on cardiovascular function is in need of extended study as suggested by Hamilton (15).

STATISTICAL INTERRELATIONS OF BODY AND ORGAN WEIGHTS WITHIN INDIVIDUAL TREATMENT GROUPS

The definition of the interrelations of the endocrines is still in process of formation and the relation of weight to function is itself open to several interpretations. In the hope, however, of finding further confirmation of our findings, the intercorrelations between body weight, liver, heart, testes, adrenals, prostate and seminal vesicles, and pituitary weights have been computed, and are given in table 4. Statistically significant correlations are indicated by an asterisk, those of high significance by bold-faced type. The criteria were 5 per cent and 1 per cent levels of significance (16).

These correlations describe relations within a single group. For the ages, body weights and structures considered here, we assume that no absolute or relative involution, except as a result of treatment, is likely. Support for this assumption is readily found in the absence of negative correlations in the 83° *temperature group* of normal controls. We are inclined to believe that negativity may, in many cases, indicate a selective suppression or elevation of a given function as a result of experimental procedures.

An examination of table 4 shows that each group of animals is represented by 21 intercorrelations involving 7 variables. Considering signs alone, 12 (slightly more than 10 per cent) of a total of 105 coefficients for the five 120-day groups are negative. At the age of 210 days the number of negative coefficients was increased to 24 or approximately 25 per cent of the total. This increase in negativity probably represents an approach to a final constitutional equilibrium under these particular experimental conditions. When the coefficients are very small they may represent a situation in which full development has been attained in only one structure of a correlated pair. When the negative value is large, however, a relative involution seems indicated.

A tabulation of the frequency of the negative correlation coefficients for each group of rats shows that at both 120 and 210 days of age negative values occur most frequently in the groups given unusual treatment, and which in aortic tests give tensility curves displaced toward senile values (table 5).

This finding becomes more significant when it is recalled that animals of the 95° *temperature group* were the lightest at 210 days while those of the *disturbed group* were heaviest. It is of interest that the data of the 83° *temperature group* (the temperature condition at which there is least call on the temperature-regulatory mechanism of the rat) has only a single negative coefficient in a total of 42.

It was then necessary to determine if this concentration of negative correlations

TABLE 4. INTER-GROUP CORRELATION COEFFICIENTS

Experiment No.	9	10	11	12	13	14	15	16	17	18	19	20	21
						Body Weight							
Age in days	120	120	120	120	120	210	210	210	210	210	120	120	120
Special Treatment	65°	83°	95°	Thy.	Dist.	65°	83°	95°	Thy.	Dist.	65°	83°	Dist.
Testes	0.372	0.552*	**0.637**	0.470*	0.287	0.508*	0.224	+0.318	−0.308	0.108	0.558*	0.551*	0.550*
Prost. and Sem. Ves.	0.151	0.201	0.359	0.431*	0.299	**0.583**	0.416	0.280	−0.011	0.219	**0.810**	**0.618**	**0.763**
Adrenals	0.549*	0.393	0.111	−0.119	−0.273	0.282	0.386	−0.435	0.181	0.369	**0.611**	0.094	**0.636**
Pituitary	0.316	0.468*	0.151	**0.625**	0.483*	**0.590**	0.499*	0.466*	0.097	−0.004	**0.847**	**0.654**	**0.744**
Liver	**0.846**	**0.659**	0.533*	**0.647**	**0.812**	0.245	**0.666**	0.427	0.553*	0.258	**0.736**	**0.837**	**0.902**
Heart	**0.888**	**0.744**	**0.612**	0.143	**0.922**	0.405	**0.863**	**0.821**	**0.720**	0.245	**0.861**	**0.871**	**0.928**
						Testes							
Prost. and Sem. Ves.	0.099	0.206	**0.571**	**0.650**	0.464*	0.247	−0.234	**0.575**	**0.631**	0.228	**0.692**	0.107	0.325
Adrenals	0.305	0.117	0.068	0.043	0.196	−0.041	0.167	−0.097	−0.222	−0.212	0.350	0.162	0.541*
Pituitary	0.450	0.001	0.204	0.363	0.407	0.087	0.392	−0.118	−0.532*	0.228	**0.635**	0.532*	0.533*
Liver	**0.622**	0.199	0.384	0.470*	0.083	−0.148	0.121	0.059	**0.481**	−0.257	0.421	0.502*	**0.584**
Heart	0.417	0.442*	0.146	0.431	0.159	0.395	**0.479**	0.123	−0.272	0.135	0.517*	0.552*	**0.588**
						Prostate and Seminal Vesicles							
Adrenals	0.090	0.116	−0.194	−0.119	0.056	0.048	0.019	0.256	0.065	−0.216	0.308	−0.087	**0.605**
Pituitary	−0.029	0.314	−0.237	**0.625**	0.262	0.509*	0.192	0.051	**−0.645**	0.149	**0.874**	0.308	**0.762**
Liver	0.130	0.069	−0.022	**0.647**	0.310	0.473*	0.376	−0.076	0.530*	0.508*	**0.665**	0.413	**0.754**
Heart	0.129	0.263	−0.043	0.143	0.168	0.468*	0.191	0.231	−0.268	**0.593**	**0.718**	0.463*	**0.788**
						Adrenals							
Pituitary	0.368	0.262	0.363	0.076	−0.091	0.165	0.359	−0.103	0.130	**0.586**	0.477*	0.482*	**0.755**
Liver	0.491*	0.498*	0.236	−0.384	−0.206	0.346	0.138	−0.055	0.105	−0.276	**0.563**	0.527*	**0.763**
Heart	0.521*	**0.631**	0.236	0.270	−0.268	0.253	0.309	−0.221	0.341	0.034	**0.691**	0.275	**0.699**
						Pituitary							
Liver	0.501*	0.459*	0.228	0.087	0.328	**0.658**	0.145	0.060	−0.414	−0.148	**0.664**	0.345	**0.738**
Heart	0.438	0.308	0.251	0.287	0.514*	**0.621**	0.484*	0.501*	0.346	0.360	**0.746**	**0.722**	**0.714**
						Liver							
Heart	**0.883**	**0.627**	0.246	0.439	**0.787**	**0.650**	**0.617**	0.280	0.387	0.312	**0.723**	**0.899**	**0.720**

* Indicates significant coefficient. Bold-faced type indicates highly significant coefficient.

TABLE 5

120 days of age			210 days of age		
Group	+r's	−r's	Group	+r's	−r's
95°	17	4	Thyroid	13	8
Disturbed	17	4	95°	14	7
Thyroid	18	3	Disturbed	15	6
65°	20	1	65°	19	2
83°	21	0	83°	20	1
Total	93	12	Total	81	24

in the groups characterized by senile-like aortic tensility curves is paralleled by a relative concentration of the same correlations in certain groups of organs when classified according to the seven structures studied. These data are given in table 6.

At 120 days of age, adrenal and prostatic weights account for 15 of 30 negative coefficients. The major incidence of negativity involving the prostate occurs in the 95° *temperature group* while all but one involving the adrenals is attributable to the *thyroid* and the *disturbed groups*.

The absolute adrenal weights in these two groups were large, but not as large as those of the 83° or the 95° *temperature groups*, when considered relative to body weight. This fact would point to the conclusion that the accelerated growth rate induced by thyroid feeding or by a disturbed environment tends to remove the normally positive correlation between adrenal and body weights. At 210 days of age the positive relation has been restored in both the *thyroid* and the *disturbed groups* (table 4). This reversal in rapidly growing animals may be interpreted as a temporary exhaustion of the adrenals in the case of the *disturbed group* and as a general visceral hypertrophy without thyroid or adrenal enlargement due to thyroid feeding. Cameron (17) noted this sparing action on the thyroid which suggested the above interpretation in view of the synergistic action of the thyroid and adrenal systems.

Negative correlation coefficients involving prostatic relationships with adrenals and pituitary in the 95° *temperature group* are probably related to the known depressant action of a hot environment on the reproductive system of the rat. At 120 days of age the prostate had attained only about 60 per cent of its final weight while the pituitary had reached almost 100 per cent of its 210-day weight and the adrenal weights were actually regressing.

At 210 days of age, as has been noted from tables 5 and 6, there is an increase in the incidence of negative correlations over that observed at 120 days. The *thyroid, disturbed* and 95° *temperature groups* again account for the majority of the negative

TABLE 6

120 days of age			210 days of age		
Structure	+r's	−r's	Structure	+r's	−r's
Adrenals	22	8	Testes	19	11
Pros. and Sem. Ves.	23	7	Adrenals	20	10
Liver	27	3	Pituitary	23	7
Pituitary	27	3	Liver	23	7
Body weight	28	2	Pros. and Sem. Ves.	24	6
Heart	28	2	Body weight	26	4
Testes	30	0	Heart	27	3
Total	185	25	Total	162	48

correlations (21 of 24) and the distribution according to structure is quite similar with one exception. At 120 days of age no negative correlations involving the testes are found while at 210 days over one-third of the testicular coefficients are negative.

These results must be interpreted in the light of at least one fundamental difference between the 120 and 210-day groups. At the latter period growth curves for all groups are leveling off, body weight-growth rate is reduced, and adult plateaus are being attained. Hence correlations between body, organ, and gland weights come closer to reflecting the constitutional characteristics of each group than at 120 days of age.

In table 2 it is seen that the average absolute testicular weights of the three *temperature groups* show a gain from 120 to 210 days of 0.14 to 0.23 grams whereas the *disturbed* and *thyroid groups* show a loss of 0.06 to 0.20 grams. The 95° *temperature group* showed a gain, but the absolute weight for the testes was the smallest of the five groups at 210 days of age. In terms of weight per gram of body weight, the *thyroid disturbed*, and 95° *temperature groups* show smaller testes than the 83° or 65° *temperature groups*. Since the poorest tensility curves were found in the *disturbed* and the 95° *temperature groups*, the fact that in the unfavorable treatment groups testicular development is retarded in both absolute and relative weight terms at 210 days is noteworthy and independent of the general level of growth which is high in the *thyroid* and *disturbed group* and low in the 95° *temperature group*. The greatest concentration of negative correlations involving the testes occurs in the *thyroid group*. As has been noted in the *thyroid group* there was actually a regression in average testicular weight between 120 and 210 days. Whether of direct or indirect nature, there is an association between testicular development and aortic tensility properties which is independent of age or weight.

Adrenal weights at 210 days of age are correlated negatively with other weights in 10 of 30 instances. Only one of six possible adrenal coefficients is positive in the 95° *temperature group* and three of six in the *disturbed group*. Just as the *disturbed* and the 95° *temperature groups* evidenced atypical testicular development by all criteria so they exhibited frequent atypical adrenal interrelations. The animals of the *disturbed group* show large gains in adrenal weight between 120 and 210 days while the reverse is true of the 95° *temperature group*. This effect of excitement and high temperature emphasizes the necessity of interpreting a high frequency of negativity in the intercorrelations as an indication either of an increase or decrease in relative development, the actual direction becoming apparent only upon inspection of data.

HISTOLOGICAL EXAMINATION

At the time of autopsy, sections from the thoracic aorta and the whole heart were preserved for histological examination. These were fixed for several days in Bouin's solution and then removed to 70 per cent alcohol. Sample paraffin sections were cut from each heart and aorta.[2] Half of the sections from each specimen were stained with hematoxylin and eosin, the other half with Mallory's connective tissue stain. Since the investigation was concerned with possible correlations between the tensility differences and histological observations, these two stains could be expected to reveal abnormalities of the tissue elements involved. The heart and aorta from five animals of each group were examined. No gross pathological changes had taken place in any of the specimens examined, and it was impossible to find a criterion which could be used to distinguish the control from the experimental groups.

In all of the specimens of the aorta, the tunica interna and tunica media presented

[2] Dr. H. W. Ades was responsible for the histological examination.

a normal appearance with no observable deficiency of any of the tissue elements. The normal proportions of elastic and collagenous connective tissues and muscular elements were undisturbed. The adventitia was badly torn in many specimens and poorly preserved, however, this is not unusual in sections of normal blood vessels.

It was thought that a comparison of the relative thicknesses of tunica interna and tunica media might be of value. Consequently measurements were made with an ocular micrometer at a magnification of 100×. Four standard locations were taken on each section and a total of eighty measurements made on each aorta. Measurement of the tunica interna was thought to be unreliable because of the possibility of distortion in this thin coat in sectioning. An analysis of 1,500 measurements of the tunica media indicated that a range of approximately ±10 per cent from the mean of the whole group would include the submeans of all groups. Obviously such small differences could not be of any considerable significance in view of the impossibility of measuring exactly comparable levels of section and because of some effect of animal size. It was necessary to conclude that there were no gross pathological changes and no microscopically observable differences in vascular structure between groups receiving different treatments.

SUMMARY AND CONCLUSIONS

In earlier contributions, it has been shown that the tensility curve obtained by stretching the thoracic aorta of the rat on the Hume tensometer is affected by age and weight. At standard age or weight, thyroid feeding, a cage temperature of 95°F. or constant sensory stimulation displaces such curves in the direction associated with increasing age (decreasing tensile resistance). Cage temperatures of 65°F. displace the curve toward values associated with younger animals (higher tensile resistance). Animals living at 83°F. give curves lying in an intermediate group.

Data are given describing characteristic growth phenomena for each experimental condition. In addition to body weight and length measurements, the weights of liver, heart, testes, adrenals, prostate and seminal vesicles, and pituitary for each animal were recorded. Summary tables are presented, giving average body, organ and gland weights for each group. The effects of treatment on gland and organ weights in relation to each other and in relation to body weight are discussed. Graphic comparisons have been made by expressing body, organ and gland weights in terms of a percentage of the 83° *temperature group*, which was considered the most direct type of control. Similar pattern effects were obtained by plotting data for the high and low environmental temperature groups together and the otherwise stimulated (thyroid feeding and disturbance) groups together. These two patterns are discussed in relation to treatment and atypical results explained as nearly as possible in the light of present knowledge of endocrine balance. A statistical treatment of the interrelations of body and organ weights within individual treatment groups is presented, as well as the results of an histological examination of the thoracic aorta and whole heart.

Marked differences in rate of growth and in mature body weight are associated with the different treatments to which five groups of male albino rats were subjected. These growth differences, however, do not provide a satisfactory explanation of the effect on aortic tensility since senile-like displacements may occur in the heaviest and lightest groups at a standard age.

Total energy production does not provide a satisfactory explanation of the curve displacement, as metabolism is elevated in the 65° *temperature group* (high resistance to stretch), the *thyroid group*, and the 95° *temperature group* (low resistance).

The tensile resistance of the rat's thoracic aorta is affected, independently of age or weight, by chronic exposure of the animal to high or low temperatures, thyroid feeding, or continued sensory stimulation.

Histological examination of the heart and thoracic aorta was negative for gross or microscopic pathology, or any disturbance of normal structure. Although no structural basis for the effect is histologically obvious, a dystrophy in endocrine development, as measured by organ and gland weights, is associated with these functional changes in the aorta.

An analysis of the weights of the liver, heart, testes, adrenal, prostate and seminal vesicle, and pituitary indicates a general tendency to distortion of normal endocrine relations in groups characterized by low aortic tensility. When body, organ and gland weights of each group are plotted as percentages of the corresponding weights of the 83° *temperature group*, two patterns are discovered, one typical of temperature effects (65° and 95°) and the other typical of stimulation effects (thyroid feeding and disturbance).

Small testicular and large prostatic and pituitary weights were the results of high or low temperature, the animals of the 95° temperature group having, in addition, very small adrenals. This endocrine pattern of the 95° group, together with the aortic properties found, is distorted in a manner normally associated with an age effect.

The most characteristic differences in the patterns of the disturbed and the thyroid groups are the small pituitaries of the former and the large hearts of the latter. Decreased weights of the pituitary, increased weights of the adrenals and decreased weights of the testes of these groups in relation to the 83° temperature group suggests an androgenic effect of adrenal cortical origin.

A review of the correlation coefficients of body, organ and gland weights reveals a fundamental difference between the 120 and the 210-day results, due to differences in rates of growth at the two age levels. Correlations between body and organ weights tend to reflect more clearly constitutional differences within each group at 210 days of age than at 120 days. At both 120 and 210 days the largest number of negative coefficients occur in the groups given unfavorable treatment (*disturbed, thyroid* and 95° *temperature groups*) and which, in aortic tests, give tensility curves displaced toward senile values.

There is a consistent association of retarded testicular development and senile-like aortic tensility curves. The fact that three distinct types of treatment (high temperature, thyroid feeding, and disturbance) produced similar gonadal effects, associated directly with similar aortic effects, points to a relationship in which the gonadal principles influence cardiovascular function.

REFERENCES

1. Hume, J. F.: *Am. J. Hyg.* 29: 11. 1939.
2. Nelbach, J. H., and L. P. Herrington: *Am. Heart J.* 22: 661. 1941.
3. Donaldson, H. H.: The Rat. Memoirs of Wistar Institute of Anatomy and Biology. No. 6. Philadelphia, 1924.
4. Kleiber, M., and J. E. Dougherty: *J. Gen. Physiol.* 17: 701. 1934.
5. Alpern, D.: *Am. J. Physiol.* 90: 264. 1929.
6. Cannon, W. B., H. F. Newton, E. M. Bright, V. Menkin and R. M. Moore: *Am. J. Physiol.* 89: 84. 1929.
7. Moore, C. R.: *Am. J. Anat.* 34: 269. 1924.
8. Herrington, L. P.: *Am. J. Physiol.* 129: 123. 1940.
9. Hill, R. T.: *Endocrinology* 21: 633. 1937.
10. Cameron, A. T.: Recent Advances in Endocrinology. Blakiston Company, Philadelphia, 1940.
11. Selye, H.: *Am. J. Physiol.* 123: 758. 1938.
12. McEuen, C. S., H. Selye and J. B. Collip: *Lancet* 1: 775. 1936.
13. Vidgoff, B., and H. Vehrs: *Endocrinology* 26: 656. 1940.
14. Vidgoff, B., and R. Hill: *Endocrinology* 25: 568. 1940.
15. Hamilton, J. B.: *Anat. Rec.* 79: 30. 1941. Abstract Supplement No. 2.
16. Fisher, R. A.: Statistical Methods for Research Workers. Oliver and Boyd, London, 1934.
17. Cameron, A. T.: Abstracts of Communications to the Eleventh Internat. Physiol. Congress held at Edinburgh, July 23–27, 1923.

THE ACTION OF CYSTEINE AND OTHER REDUCING AGENTS UPON PREGNANT-MARE-SERUM GONADOTROPIN

JOHN S. EVANS, JOHN W. NELSON AND GEORGE F. CARTLAND

From the Research Laboratories, The Upjohn Company

KALAMAZOO, MICHIGAN

THE ACTION OF CYSTEINE upon gonadotropins has been studied by two groups of workers (1, 2, 3) with regard to the relationship between the disulfide linkage and the activity of gonadotropins. Fraenkel-Conrat, et al. (1) claimed that cysteine did not produce inactivation of pregnant-mare-serum preparations or prolan, but did inactivate pituitary follicle-stimulating hormone and leuteinizing hormone. Bischoff (2) reviewed the data of Fraenkel-Conrat, et al., and regarded their work as inconclusive because of their failure to take into consideration the question of a possible change in the resorption rate, especially with respect to the pituitary hormones.

Fraenkel-Conrat, et al. (3) reported that when the protein concentration of the solution was adjusted to 1 mg. per cc., pregnant-mare-serum gonadotropin was inactivated by cysteine. Their earlier reported work was made on a more dilute solution. They attributed the inactivation to the presence in the molecule of disulfide linkages of intermediate reactivity. When the hormone (pregnant mare serum) was allowed to react for a longer period of time or in the presence of 40 per cent urea, which is known to produce denaturation and render the -S-S- groups more reactive, inactivation did occur upon treatment with cysteine or thioglycollic acid. "Since no protein groups besides -S-S- bonds have been shown to react with such compounds, the assumption appears justified that the reduction of certain of these bonds causes the observed inactivation of gonadotropins" (3). This is in apparent contrast to the statement of Fleischer, et al. (4) who state that the "gonadotropic hormones apparently do not contain cystine."

Fraenkel-Conrat, et al. (1) have reviewed the literature relating to the use of cysteine as a means of studying the stability of the -S-S- linkages as well as that on how the biologically active proteins may differ in their reaction to cysteine in mildly alkaline solution.

In view of the controversial aspect of the action of cysteine upon pituitary gonadotropins, it was decided to repeat the work of Fraenkel-Conrat, et al. upon pregnant mare serum and to apply other reducing agents to the hormone.

EXPERIMENTAL PROCEDURE

The assay of the hormone and the reaction products was conducted by the method of Cartland and Nelson (5) using 21 to 23-day-old female rats. The results of the assays are expressed in terms of international units (I.U.).

The starting material was a purified fraction (*41-HPA-7*) assaying 800 I.U. per mg. and a fraction (*250-HPA-7*) assaying 1,800 I.U. per mg.

The action of cysteine. Pregnant mare serum (*41-HPA-7*) was dissolved in 2 per

Received for publication, January 26, 1942.

cent butanol. An amount of cysteine-*HCL* equivalent to forty times the weight of pregnant mare serum used was added to the solution. The pH was adjusted to 8.0 by the use of NaOH, and the volume made up with 2 per cent butanol to give a solution containing 1 mg. per cc. A similar solution of pregnant mare serum at pH 8.0, but without the cysteine-HCl, served as a control. The two solutions were stored in glass-stoppered bottles with only a very small air space, at room temperature (25–27°C.) for 44 hours. The results are shown in table 1.

TABLE 1. EFFECT OF CYSTEINE UPON PREGNANT MARE SERUM

Materials and Treatment	Assay Level	Ovarian Weight	Assay	Per Cent Inactivation
	I.U./mg.	mg.	I.U./mg.	
PMS in 2% BuOH at room temperature for 44 hours	800	59	740	None
	800	80	920	None
	600	121	880	None
PMS in 2% BuOH plus cysteine at pH 8.0, room temperature for 44 hours	800	14		100
	600	16		100
	200	15		100
Uninjected controls		15		

The results indicate that cysteine is capable of inactivating pregnant mare serum when the proper protein concentration is used. This agrees with the conclusions reached by Fraenkel-Conrat, et al. (3).

The action of cyanide on pregnant mare serum. Maxwell and Bischoff (6) found that there was no detectable loss of activity when pituitary preparations were treated with hydrogen cyanide. Cyanide has been used extensively in analytical practice for the reduction of disulfide bonds. We have tested the effect on the activity of pregnant mare serum of cyanide by itself and in the presence of urea.

To 3 mg. of pregnant mare serum dissolved in 2.5 cc. of buffer at pH 8.0, 40 mg. of NaCN was added. This was allowed to stand for the desired period of time. The pH was adjusted to 6.0 with HAc. A few drops of I_2 was added to remove the cyanide. Excess iodine was then removed with thiosulfate. In the experiments involving urea, sufficient urea was added to make the solution 40 per cent. The exposure to iodine was made as brief as possible.

The results show that cyanide produces some inactivation within a period of 30 minutes and was very evident after 60 minutes' exposure. This is in contrast to Bischoff and Long's (7) experience with prolan which they found to be stable to cyanide for a period of one hour at pH 6.5. Bischoff considers that the inactivation of pituitary

TABLE 2. EFFECT OF CYANIDE ON PREGNANT MARE SERUM

Materials and Treatment	Assay Level	Ovarian Weight	Assay	Per cent Inactivation
	I.U./mg.	mg.	I.U./mg.	
PMS	800	56	720	None
PMS and NaCN (20 min.)	800	58	740	None
PMS and urea (20 min.)	800	34	540	25
PMS, NaCN and urea (20 min.)	600	26	300	58
PMS and NaCN (30 min.)	800	29	260	64
PMS and NaCN (60 min.)	800	21	140	82
PMS, NaCN and urea (30 min.)	800	16	<80	>90
PMS, NaCN and urea (60 min.)	800	14	<80	>90

gonadotropin (s) and prolan produced by large excesses of cysteine or cyanide and long periods of reaction may not be due to their action on the disulfide linkages, but to side reactions. The same criticisms may be applied to the work on pregnant mare serum.

Effect of other reducing agents on pregnant mare serum. Pregnant mare serum (pH 7.27) was not inactivated upon exposure to H_2S for periods up to one hour. If the exposure to H_2S was made in a medium containing 40 per cent urea, there was evidence of about 50 per cent inactivation.

Pregnant mare serum was not found to be inactivated by nascent hydrogen (Mg filings and weak acetic acid). In the presence of urea a slight loss of activity occurs but this loss may be due to animal variation in the assay method.

Pregnant mare serum was dissolved in a phosphate buffer (pH 7.3) to give a concentration of 1 mg. per cc. Glutathione was added to give a concentration of 10 mg. per cc. Ascorbic acid was added to give a concentration of 4 mg. per cc. No reduction took place when the mixture was allowed to stand as long as 60 minutes at room temperature. After one week in the refrigerator, about 82 per cent of the activity was lost, whereas the pregnant-mare-serum control showed no loss of activity.

Table 3. Effect of reducing agents on pregnant mare serum

Material and Treatment	Assay Level	Ovarian Weight	Assay	Per cent Inactivation
	I.U./mg.	mg.	I.U./mg.	
PMS	800	62	780	None
PMS and H_2S (15 min.)	400	158	760	None
PMS, urea and H_2S (60 min.)	400	76	440	45
PMS in HAc, pH 5.7 (60 min.)	800	114	1140	None
PMS, HAc and Mg, pH 5.7 (60 min.)	800	94	1020	10[1]
PMS, HAc and urea (40% solution), pH 6.7 (60 min.)	800	90	1000	10[1]
PMS, HAc, Mg and urea (40% solution), pH 6.7 (60 min.)	400	198	>840	<16
PMS	800	65	800	None
PMS and ascorbic acid	600	21	140	82
PMS and glutathione (3 hours)	600	107	820	None
PMS after one week in the refrigerator	800	85	960	None
PMS and glutathione after one week in the refrigerator	300	138	480	50

[1] Probably insignificant.

Pregnant mare serum was dissolved in phosphate buffer (pH 7.3) to give a protein concentration of 1 mg. per cc. No inactivation was observed at the end of 3 hours. After 18 hours at room temperature about 50 per cent inactivation was noted. After standing for one week in the refrigerator no further inactivation occurred.

Nitroprusside reaction. A purified preparation of pregnant mare serum, assaying 1,800 I.U. per mg., was tested for -SH and -S-S- groups by the nitroprusside reaction. Fifteen mg. of the material was dissolved in a volume of H_2O small enough that the final solution would be only 1 cc. The reaction was negative. Similar samples were denatured by boiling in an acid solution and by using urea (40% solution), with negative nitroprusside results. Treatment with cyanide led to negative results. The sensitivity of the reaction is only about 1:10,000 (8). The test was then made using the method of Mirsky and Anson (9). About 50 mg. of the 800 I.U. of pregnant mare serum was

placed in an evaporating dish. A few crystals of NaCN were mixed with the pregnant mare serum and the mixture moistened with a few drops of 5 per cent sodium-nitroprusside solution. The protein material turned red, indicating the presence of disulfide groups.

Sulfur determination. A sulfur determination was made on a fraction of pregnant mare serum assaying 800 I.U. per mg. and two other fractions assaying 1,800 I.U. per mg. In order to obtain a sulfur determination it is necessary to use nearly 100-mg. samples. It was characteristic of all determinations that the $BaSO_4$ came down as very small crystals and it was necessary to take much care in removing the precipitate. The results of the sulfur determinations are shown in table 4.

TABLE 4. SULFUR CONTENT OF PREGNANT MARE SERUM

Fraction	Assay	Sulfur	Calculated Cystine	Weight of Sample	Weight of $BaSO_4$ Found
	I.U./mg.	%	%	mg.	mg.
41-HPA-7	800	0.50	1.87	126.93	4.629
250-HPA-7	1800	0.655	2.46	87.60	4.178
13-HPA-8	1800	0.854	3.09	102.81	6.373

Papp (10) showed that the average cystine content of the total protein of horse serum was 2.86 per cent of the dry matter. The average cystine content of the proteins separated by $(NH_4)_2SO_4$ precipitation was 1.96 to 2.78 per cent. If one calculates the sulfur present as cystine, one finds that the calculated concentration of cystine lies in the same range that Papp (10) found for the proteins separated by $(NH_4)_2SO_4$.

A small quantity of the hormone was dialyzed against distilled water to remove any dialyzable sulfate that might be present. The hormone was then precipitated by acetone and analyzed for sulfur. No significant change in the sulfur content was noted. Since there was no H_2SO_4 used in the preparation of these fractions, we feel that the sulfur is an integral part of the hormone.

DISCUSSION

Fraenkel-Conrat, et al. (3) state that cysteine decreases or destroys the activity of all of the gonadotropins which they have studied (pituitary follicle-stimulating hormone, prolan, pregnant mare serum and samples prepared from teratoma testis urine). In this laboratory, we have found that cysteine and cyanide inactivate pregnant mare serum. Other reducing agents inactivate the hormone to a more or less degree.

The general opinion of investigators (11, 12, 13) is that the action of thiol compounds, i.e. cysteine or thioglycollic acid, on proteins is to reduce the disulfide linkages. du Vigneaud (11) showed that low concentration of cysteine would inactivate insulin, but that the reaction time is much longer.

Fleischer, et al. (4) have expressed the view (but do not give data) that pregnant mare serum does not contain any cystine. The biological response of pregnant mare serum indicates the presence of disulfide linkages in the molecule. We have obtained a positive nitroprusside reaction with a highly active pregnant-mare-serum fraction, indicating the presence of a disulfide linkage in the molecule.

The failure of other workers to find sulfur or cystine in pregnant mare serum is probably due to their failure to use enough material for their determination. However, there is the possibility that Fleischer, et al. (4) have much purer material from which the sulfur-containing component has been removed.

SUMMARY

Cysteine and NaCN are capable of inactivating pregnant-mare-serum gonadotropin.

Hydrogen sulfide and nascent hydrogen do not inactivate pregnant mare serum alone under the conditions used. Hydrogen sulfide in the presence of 40 per cent urea produces 60 per cent inactivation. Nascent hydrogen did not affect pregnant mare serum in 40 per cent urea solution.

A purified preparation of pregnant mare serum gave a positive nitroprusside reaction indicating the presence of a disulfide group in the molecule.

Preparation of pregnant mare serum of two grades of purity showed 0.50 and 0.85 per cent sulfur. If the sulfur content is calculated as cystine, the cystine content would agree with that found by Papp (10) for horse serum proteins.

REFERENCES

1. Fraenkel-Conrat, H., M. E. Simpson and H. M. Evans: *J. Biol. Chem.* 130: 243. 1939.
2. Bischoff, F.: *J. Biol. Chem.* 134: 641. 1940.
3. Fraenkel-Conrat, H., M. E. Simpson and H. M. Evans: *Science* 91: 363. 1940.
4. Fleischer, G., E. Schwenck and K. Meyer: *Nature* 142: 835. 1938.
5. Cartland, G. F., and J. W. Nelson: *Am. J. Physiol.* 122: 201. 1938.
6. Maxwell, L. C., and F. Bischoff: *J. Biol. Chem.* 112: 215. 1935.
7. Bischoff, F., and M. L. Long: *J. Biol. Chem.* 116: 285. 1936.
8. Walker, E.: *Biochem. J.* 19: 1082. 1925.
9. Mirsky, A. E., and M. L. Anson: *J. Gen. Physiol.* 19: 439. 1935.
10. Papp G.: Közlemények az összehasonlító élet és kórtan köréből 27: 232. 1940. Chem. Abstracts 24: 2433[2]. 1940.
11. du Vigneaud, V., A. Fitch, E. Pekaret and W. W. Lockwood: *J. Biol. Chem.* 94: 233. 1931.
12. Mirsky, A E., and M. L. Anson: *Proc. Soc. Exper. Biol. & Med.* 28: 170. 1930.
13. Wintersteiner, O.: *J. Biol. Chem.* 102: 473. 1933.

UNSUITABILITY OF THE MOUSE-UTERINE-WEIGHT METHOD FOR ASSAY OF CHORIONIC GONADOTROPIN

JAMES D. HAUSCHILDT AND JOHN S. EVANS
From the Research Laboratories of The Upjohn Company
KALAMAZOO, MICHIGAN

USE OF THE MOUSE as a test animal for the assay of gonadotropic substances from urine has been considered by a number of workers (1–5). Evans et al. (1) obtained good results when applying the method only to assay of estrogens; Hamburger and Pedersen-Bjergaard (2) presented data showing good agreement between assays from two laboratories using different strains of mice and the same pregnancy urine gonadotropic preparation. Levin and Tyndale (3) found the mouse-uterine-weight method satisfactory for the assay of follicle-stimulating substances from castrate and postmenopausal urine but stated that the method is not so accurate for assay of the hormone from pregnant mare's serum or human pregnancy urine. They gave no data, however, on the assay of pregnant mare's serum hormone or the human pregnancy urine hormone.

Preliminary work on both our own laboratory standard and on the International Standard of chorionic gonadotropin indicated that the method might be applied successfully to the assay of pregnancy urine gonadotropic preparations. Results which are presented below did not bear out our hope that the method would be feasible.

EXPERIMENTAL

During the course of this work two strains of mice each from a different mousery were used. The history of one strain is uncertain; the other has been inbred for 40 generations and should therefore be uniform. Numerous known pregnancy urines were assayed in addition to the repeated assay of many preparations of varying degrees of purity prepared by three different methods. For the purposes of this paper, however, it is sufficient to present results obtained by use of the International Standard preparation and a laboratory standard of fairly high potency.

Assays were carried out according to the method of Levin and Tyndale (3). Mice were albino virgin females weighing 6 to 8 gm. at the beginning of the experiment. Uteri were weighed on a torsion balance after dissecting, pressing dry, and stripping off the fat and mesentery. At autopsy the vagina was recorded as open or closed. As a rule, 7 mice on each dosage level were used for any one assay. Controls usually numbered 7 to 10. Occasionally more but never less than 7 mice were used on a level. The total dose given to each mouse was 0.3 cc. or 0.1 cc. per injection. The summary of the results obtained on the standards is shown in table 1.

We attempted to choose the number and spacing of dosage levels in such a way that there would be several small increases over the range in which the uterine weight shows its greatest sensitivity to a change of dosage, i.e., the sharply ascending portion of the dose-response curve.

Received for publication December 22, 1941.

TABLE 1. ASSAY OF CHORIONIC GONADOTROPIN ON MOUSE UTERUS

Date	No. of mice	I.U. to give 150% increase in uterine wt.	Preparation	Range in I.U. given	No. of dosage levels given
4/10/41	100	0.3	℞ 1501	0.01–0.4	10
4/21/41	60	0.25	℞ 1501	0.25–0.45	5
4/22/41	50	0.3	Int. Std.	0.25–0.45	5
4/25/41	22	0.3	Int. Std.	0.3 –0.4	3
5/15/41	30	<0.3	Int. Std.	0.3 –0.45	4
6/ 2/41	41	0.2–0.25	℞ 1501	0.1 –0.3	5
9/ 5/41	28	>0.25	℞ 1501	0.1 –0.25	4
9/15/41	28	>0.35	℞ 1501	0.2 –0.35	4
9/20/41	90	>0.45	℞ 1501	0.2 –0.45	6
9/20/41	42	>0.35	Int. Std.	0.1 –0.35	6
9/22/41	21	>0.35	Int. Std.	0.25–0.35	3
9/22/41	28	0.25–0.3	℞ 1501	0.2 –0.35	4
10/27/41	42	>0.7	Int. Std.	0.2 –0.7	6
10/27/41	42	>0.7	℞ 1501	0.2 –0.7	6
11/ 4/41[1]	42	0.45	℞ 1501	0.2 –1.2	6
11/16/41[1]	35	0.25	℞ 1501	0.2 –0.6	5
12/ 1/41[1]	245	>0.6	℞ 1501	0.1 –0.6	6

[1] Changed strain of mice.

In order to establish first the sensitivity of a strain of mice to prolan, as on April 11, and Nov. 4, 1941, 6 to 10 dosage levels over a wide range were used. After noting the effective range, the number of dosage levels was limited to a more narrow range in an attempt to have a negative response, a response on the steep portion of the curve, and a response on the plateau portion of the curve in each group of assays.

The variation in the response of the mice from week to week (note the last three assays in table 1) makes it very difficult to assay the hormone with any degree of accuracy unless larger numbers of groups are used on each assay. If the spread of dosage between the groups was increased, then the accuracy would likewise be less.

As may be seen, the results obtained at the first part of the work were encouraging and appeared consistent. The data in table 2 are a more detailed example of the results which may sometimes be obtained. The response is seen to be consistent with the dosage if only uterine weight is considered. We have found a complete lack of correlation at all times between opening of the vagina and the amount of gonadotropin given; values in table 2 are an example.

TABLE 2. ASSAY OF CHORIONIC GONADOTROPIN ON MICE

I.U./mouse	Number of mice	Average uterine weight	Average body weight[1]	Vaginas opened
		mg.	*gm.*	%
0.0	10	7.17	11.5	0
0.04	10	8.09	10.5	10
0.08	9	8.74	9.0	0
0.20	9	10.14	9.7	0
0.30	9	14.81	11.0	67
0.40	10	20.83	10.8	30

[1] Body weight at end of experiment.

Later in the work the animals appeared to lose sensitivity and the assays of unknowns as well as standards became difficult to interpret. Even when positive responses were obtained a lack of consistency was apparent between the amount of hormone given and the weight of the uteri. In other words, the typical sigmoid dose-

response curve obtained so frequently with the organ-weight method of assay was not always obtained. A typical example is given in table 3.

TABLE 3. ASSAY OF CHORIONIC GONADOTROPIN ON MICE

Dose per mouse	Number of mice	Average uterine weight	Average body weight[1]
I.U.		*mg.*	*gm.*
0.0	7	5.2	8.0
0.2	7	8.1	8.6
0.3	7	19.1	9.9
0.4	7	10.2	9.0
0.5	7	10.4	8.1
0.6	7	17.5	9.3

[1] Body weight at end of experiment.

In such a case as this the mouse unit was taken as between 0.2 and 0.3 I.U. We are at a loss to explain the values obtained with 0.4 and 0.5 I.U. Frequently the same kind of response was observed when unknown preparations were given. We were thus forced to abandon the method, since we felt that with a total of 950 mice on the two standards, sufficient data were on hand to justify the conclusion that the method is unsuitable for the assay of the chorionic gonadotropin.

DISCUSSION

It is difficult to reconcile the results presented here with those of Hamburger and Pedersen-Bjergaard (2) whose data show a remarkable consistency between response and dosage and between strains of mice. However, the large number of animals used by us would seem to indicate that our results are not due to chance. As we have shown, in any one assay using 7 to 10 mice on each dosage level, a good dose-response curve may be obtained; however, the week to week response to a given dose is so variable when tested on a large number of mice that in our opinion the method cannot be applied to the assay of pregnancy urine preparations of unknown potency with any degree of accuracy, even though standards are run simultaneously.

SUMMARY

Data obtained from 950 mice are presented showing that no constant dose-response relationship exists between the gonadotropic hormone of human pregnancy urine and the uterine weight of mice from two different strains. It is concluded that the mouse-uterine-weight method is unsuitable for the assay of this hormone.

REFERENCES

1. EVANS, J. S., R. F. VARNEY, AND F. C. KOCH: *Endocrinology* 28: 747. 1941.
2. HAMBURGER, C., AND K. PEDERSEN-BJERGAARD: *Quart J. Pharm. & Pharmacol.* 10: 662. 1937.
3. LEVIN, L., AND H. H. TYNDALE: *Endocrinology* 21: 619. 1937.
4. KATZMAN, P. A., AND E. A. DOISY: *J. Biol. Chem.* 98: 739. 1932.
5. ROWE, L. W., A. SIMOND AND W. O. NELSON: *J. Am. Pharm. A.* 23: 882. 1934.

CHANGES IN HORMONE CONTENT OF THE FEMALE RABBIT HYPOPHYSIS AFTER MATING

JOHN A. SAXTON, JR., AND HARRY S. N. GREENE

From the Rockefeller Institute for Medical Research
Department of Animal Pathology
PRINCETON, NEW JERSEY

EXPERIMENTS cited by Hill (1) have shown that in the rabbit ovulation is brought about by a neuro-secretory mechanism. Impulses induced by copulation stimulate the anterior hypophysis to discharge gonadotropic hormones, which may then act upon the ovary to produce ovulation in from ten to twelve hours. Ovulation will not take place if the hypophysis is removed within one hour after mating (2). Hill (1) has shown that there is a progressive decrease in gonadotropic potency of the rabbit hypophysis following mating, and that the lowest level of potency is reached in about 24 hours. The discharge from the hypophysis would thus appear to continue beyond the period required for ovulation. The hormones released in the postovulatory period may therefore be available for the production of corpora lutea and perhaps for other processes. Hill's method of analysis, using the female rabbit in estrus as the test animal, gives a measure of the gonadotropic potency only, and does not permit an estimation of the relative amounts of other hormones discharged simultaneously.

The method of Loeb (3), using the immature female guinea pig as the test animal, enables an assay of the content of gonadotropic (follicle-stimulating and luteinizing), thyrotropic and adrenal cortex-stimulating hormones of the rabbit hypophysis (4). This method has been used in a study of the hypophyses of mated female rabbits in order to determine indirectly the composition of the discharge of hormones after mating.

MATERIALS AND METHODS

The hypophyses were obtained from 7 female rabbits of a Belgian-English hybrid strain, aged 4 to 6 months, and killed 4 to 48 hours after mating. Ovulation was confirmed at autopsy in all but one animal, which was killed 4 hours after mating. In this case, congestion of the ovaries and enlargement of the follicles was taken as evidence that ovulation would have occurred at the expected time.

The method of analysis has been described in detail elsewhere (4). Five hypophyses, from rabbits killed 4, 24, 24, 28 and 48 hours after mating, were analyzed separately by subcutaneous implantation of one-quarter of the gland daily for 4 days into immature female guinea pigs. One experiment was performed using the hypophyses of two rabbits killed 24 hours after mating, one half gland being implanted daily into a guinea pig. As a control to this experiment, the hypophyses of two unmated females, litter mates of these rabbits, were implanted in the amount of one half gland daily into a single guinea pig.

In each case the test animals were killed 24 hours after the fourth implant. At autopsy both ovaries and the left adrenal of each guinea pig were weighed, and the gross appearance of the thyroid, uterus and vagina were noted. Microscopic sections

Received for publication January 14, 1942.

of both ovaries, the left adrenal and one thyroid lobe were prepared. The standards previously described (4) were used in evaluating the changes in the test animals. Thus the effects of mating on the hormone content of the hypophysis could be determined by a comparison of these results with test animals receiving implants of single hypophyses from normal female rabbits. The average values for six untreated guinea pigs and the changes resulting from implantation of glands from normal Belgian-English females of the same age are included from the previous report for comparison.

RESULTS

The effects of implanting single rabbit hypophyses into immature female guinea pigs are shown in table 1. Hypophyses obtained within 48 hours after mating pro-

TABLE 1. EFFECTS IN GUINEA PIGS OF IMPLANTATION OF INDIVIDUAL HYPOPHYSES FROM 5 MATED FEMALE RABBITS

Experiment	Av. Wt. of Hypophysis	Av. Initial and Final Wt.	Condition of Uterus and Vagina	Ovaries			Thyroid Stimulation	Left Adrenal	
				Av. wt.	Follicle maturation	Luteinization		Av. wt.	Mitoses/section, av.
	mg.	*gm.*		*mg.*				*mg.*	
*Control, 6 normal untreated guinea pigs	—	189	Ut. thin; vag. closed	16	o	□	o	41	10
*Hypophyses from 8 normal female rabbits	39	181–181	Ut. enlarged; vag. open	29	++	+++	+	55	20
Hypophysis from 1 female 4 hr. after mating	38	175–155	Ut. enlarged; vag. open	26	+±	+	+	46	123
Hypophyses from 2 females 24 hr. after mating	38	183–190	Ut. enlarged; vag. open	22	+±	±	□	48	20
Hypophysis from 1 female 28 hr. after mating	31	180–188	Ut. enlarged; vag. open	20	+	±	□	37	18
Hypophysis from 1 female 48 hr. after mating	32	175–175	Ut. thin; vag. closed	41	+±	+	+	46	18

* These experiments are from a previous paper (4).

duced less follicle maturation and less luteinization in the ovaries of the test animals than did hypophyses from normal unmated female rabbits of the same age and strain. This indicates that both follicle-stimulating and luteinizing hormones are discharged during the postcopulatory period. The follicle-stimulating and luteinizing effects were lowest at 24 and 28 hours after mating, corresponding to the period of lowest gonadotropic potency observed by Hill (1). At 4 hours after mating there was less depletion

than at 24 or 28 hours, and at 48 hours the gonadotropic effects appeared to be partially restored. Hypophyses from mated rabbits as compared with unmated ones produced a relatively slight increase in weight of the adrenals over the average weight of adrenals in untreated guinea pigs. However, the number of mitoses in representative sections was increased in all cases, and in this respect the adrenal stimulation was similar to that found after implanting glands from normal rabbits. Single hypophyses from rabbits 24 and 28 hours after mating produced no stimulation of the thyroid, in contrast to slight or moderate stimulation usually produced by single hypophyses from normal unmated female rabbits.

To confirm the decrease in thyrotropic potency following mating, larger amounts of hypophyseal tissue were used. In table 2 are shown the effects of implanting twice the amount from mated and unmated rabbits. Hypophyses from two unmated rabbits

TABLE 2. EFFECTS IN GUINEA PIGS OF IMPLANTATION OF HYPOPHYSES FROM 2 MATED FEMALE RABBITS, AND OF HYPOPHYSES FROM 2 UNMATED FEMALE RABBITS

Experiment	Av. Wt. of Hypophysis	Av. Initial and Final Wt.	Condition of Uterus and Vagina	Ovaries			Thyroid stimulation	Left Adrenal	
				Av. wt.	Follicle maturation	Luteinization		Av. wt.	Mitoses/section, av.
	mg.	*gm.*		*mg.*					
Hypophyses from 2 unmated rabbits implanted into 1 guinea pig	49	175–200	Ut. enlarged; vag. closed	21	++	+++±	++±	46	24
Hypophyses from 2 rabbits 24 hr. after mating	47	170–185	Ut. enlarged; vag. open	31	+++	++	+	70	36

produced marked thyroid stimulation in the test animal in contrast to slight stimulation produced by hypophyses from two litter mates of these rabbits 24 hours after mating. Luteinization was greater in the ovaries of the test animal receiving hypophyses from the unmated rabbits, but follicle maturation was less. Microscopic examination of the ovaries showed that in this case the excessive premature luteinization had largely prevented follicular growth and maturation. In each case the adrenal showed evidence of stimulation, but was more marked in the guinea pig receiving the hypophyses from the mated rabbits.

COMMENT

The results so far as changes in gonadotropic and thyrotropic effects are concerned, were consistent and appear to be significant in spite of the small number of cases. These experiments confirm the observations of Hill (1), that there is a decrease in gonadotropic potency of the female rabbit hypophysis after mating. Furthermore, they show that both the follicle-simulating and the luteinizing hormones are decreased in the hypophysis of the recently mated rabbit. Hormones associated both with follicle maturation and with corpus luteum formation appear to be released from the female rabbit hypophysis through the stimulus of mating. Because this discharge continues for a period longer than that required for ovulation, it is probable that the gonadotropic hormones released become available for the subsequent production and proper development of corpora lutea. Fee and Parkes (2) and Smith and White (5)

have shown that ovulation will occur if the hypophysis is removed one hour after mating, but that the subsequent development of corpora lutea under these conditions is abnormal.

Not only gonadotropic hormones but also thyrotropic and perhaps adrenal-stimulating hormones appear to be released from the female rabbit hypophysis during the period after mating. Evidence for the release of adrenal-stimulating hormones is, however, not conclusive, and more exact methods may be required to test this observation. Mating of the female rabbit appears to produce a discharge of several hypophyseal hormones. While it is not certain that this discharge is specific, many clinical and experimental studies have shown a relationship of the thyroids and adrenals to pregnancy. It is possible therefore that the discharge of hormones other than gonadotropic is also specific, and that stimulation of the organs on which these hormones act is necessary for the initiation and proper maintenance of pregnancy in this species. Krjlow and Steinberg (6) have shown that coitus causes in female rabbits a rapid and almost complete removal of colloid from the thyroid, with associated swelling of the acinar epithelium. During the middle period of pregnancy the colloid is again restored. It seems probable that the post-copulatory discharge of thyrotropic hormone is responsible for this stimulation. In a study of the hormone content of hypophyses from old sterile rabbits (4), it was found their hypophyses contained less thyrotropic and usually more adrenal-stimulating hormones than did those from young fertile females. A balance may be necessary for the proper initiation and maintenance of pregnancy.

SUMMARY

Changes in hormone content of the female rabbit hypophysis after mating were studied by implantation of hypophyses from recently mated rabbits into immature female guinea pigs. Glands obtained from rabbits 4 to 48 hours after mating produced less follicle maturation and less luteinization in the ovaries of the test animals than did those from normal unmated female rabbits of the same age. Glands obtained 24 and 28 hours after mating produced less stimulation of the thyroid as seen in microscopic section and less stimulation of the adrenal as measured by weight increase than did those from normal unmated female rabbits. These differences indicate that follicle-stimulating, luteinizing, thyrotropic and perhaps adrenal-stimulating hormones are released from the female rabbit hypophysis after mating.

REFERENCES

1. Hill, R. T.: *J. Physiol.* 83: 129. 1934.
2. Fee, A. R., and A. S. Parkes: *J. Physiol.* 67: 383. 1929.
3. Loeb, L.: *Endocrinology* 16: 129. 1932.
4. Saxton, J. A. Jr., and H. S. N. Greene: *Endocrinology* 24: 494. 1939.
5. Smith, P. E., and W. E. White: *J. Amer. Med. Assoc.* 97: 1861. 1931.
6. Krjlow, L. N., and A. J. Sternberg: *Endokrinologie* 10: 37. 1932.

A METHOD FOR THE ASSAY OF THE GONADOTROPIN CONTENT OF NORMAL HUMAN URINE[1]

ROGER F. VARNEY AND F. C. KOCH

From the Department of Biochemistry, University of Chicago

CHICAGO, ILLINOIS

THE PURPOSE OF THE WORK reported here is to establish a reliable, simple, reproducible assay method for gonadotropic activity in the urine from normal men and women. Among the many methods which have been suggested for this purpose, the following should be emphasized. Zondek's (1) alcohol precipitation has been modified and used by Albright, Halsted and Cloney (2), Saethre (3), Heller and Heller (4), McCullagh (5), and many others. Katzman originated the use of tungstic acid (6, 7, 8) and the adsorption of the activity on finely divided benzoic acid (8, 9). The latter he later abandoned (10). Levin and Tyndale (11, 12, 13) favor precipitation by tannic acid, a method also used by Katzman (10) Friedman and Weinstein (14) and von Haam and Rothermich (15).

In preliminary work we found the tannic acid method satisfactory for human pregnancy urine, but when we attempted to use it on the much less active urine of normal women, we found that it was useless because of the high toxicity of the tannic acid precipitates. Our experience with the benzoic acid adsorption method of Katzman confirms his later conclusions (10), namely, that in this procedure there is considerable loss of gonadotropic activity (see table 1). In many cases the benzoic acid precipitates from urines of low activity were too toxic to permit assay. The alcohol-ether method originated by Zondek (1) as modified by Leonard and Smith (16) was also studied.

Our reasons for choosing the mouse as the assay animal, as explained in detail in other papers (17, 18), are based on the much greater sensitivity of the mouse uterus to the active substance and the greater uniformity of the response to increasing doses of the material. Our mouse unit is defined as the amount of activity necessary to cause a 100 per cent increase over the controls in the uterine weight of an immature (6–8 gm.) white female mouse. The material to be assayed is dissolved or suspended in 3 cc. of saline and 0.5 cc. is injected subcutaneously twice a day for three days. The test animals are killed on the morning of the fifth day, the uteri removed, freed of connective tissue and contained fluid and weighed on a torsion balance. Four or more mice are used on each dosage level. The levels injected are calculated so that a positive response in each successive dosage group of animals corresponds to 10, 20, and 30 mouse uterine units, respectively, per 24-hour samples of urines from normal males and females. In urines in which higher activity is expected the dilution is increased proportionately. This method parallels closely the mouse uterine method of Levin and Tyndale (12), but there are some differences in that they use somewhat larger mice (7–10 gm.), inject once daily for three days, and autopsy 72 hours after the first injection. Their mouse uterine unit is defined as the amount of activity causing

Received for publication January 2, 1942.

[1] These investigations were supported in part by grants from the Otho S. A. Sprague Memorial Institute and Armour & Company.

a 100 to 150 per cent increase over the control uterine weight. In spite of these differences in technic, an assay by us with a tannate sent by Dr. Levin shows that our assay agrees with his within 25 per cent and values obtained in the two laboratories on various types of urine seem to indicate that the units are approximately the same.

EXPERIMENTAL PROCEDURE

Preparation of alcohol precipitate. To the urine which was chilled and adjusted to pH 4.8 to 5.2 with acetic acid, four volumes of 95 per cent ethanol were added. The 'crude alcohol precipitate' was collected by settling and centrifugation in the freezing room. In urines from postmenopausal or castrate women in which the activity is relatively high, this crude precipitate can be injected without further treatment, but for assays on less active urines toxic substances must be removed. The results recorded in this paper clearly show the preference of the 50 per cent alcohol extraction procedure applied on this crude alcohol precipitate over the dialysis thereof and over the tannic acid precipitation process.

Recovery of added activity from urine of normal males. This portion of the work tests the recovery from urines of normal males of added active material from the

TABLE 1. RECOVERY OF ADDED GONADOTROPIN[1] FROM URINE OF NORMAL MALES BY THE ALCOHOL AND BENZOIC ACID METHODS

Aliquots and Treatment	Mouse Uterine Units per Liter	Total Mouse Uterine Units
A. Normal male urine (5 liters) precipitated with 4 volumes of ethanol. No added gonadotropin	>8	>40
B. Normal male urine (5 liters) with 133 m.u.u.[2] gonadotropin added. Precipitated with 4 volumes of ethanol	>40	>200
C. Normal male urine (5 liters) with 133 m.u.u.[2] gonadotropin added. Precipitated by benzoic acid method	2.5–5.0	12.5–25.0
D. Equivalent of 1 liter of the supernatant from *aliquot* C precipitated with 4 volumes ethanol	20	100

[1] We wish to thank Dr. J. A. Morrell of E. R. Squibb & Sons, New Brunswick, N.J., for this material (Gamone).

[2] Assayed previously by us on mice.

urine[2] of postmenopausal women by the benzoic acid method and by the alcohol method. The alcohol and benzoic acid precipitates were assayed without further treatment since the high activity makes the toxicity a negligible factor. From table 1 it is seen that the alcohol method recovers more than 200 mouse uterine units compared to the 12 to 25 M.U.U. recovered by the benzoic acid method. That the activity which is not present in the benzoic acid precipitate is partially destroyed is indicated by the fact that of the more than 200 M.U.U. shown to be present (*aliquot B*), only 12 to 25 M.U.U. are recovered in the benzoic acid precipitate (*aliquot C*) and 100 units by the subsequent alcohol precipitation of the supernatant from the benzoic acid precipitation (*aliquot D*). Thus of more than 200 M.U.U. present not more than 125 can be recovered by the consecutive application of the benzoic acid and alcohol methods on the same solution, indicating that not only does the benzoic acid method fail to recover all of the activity, but that some of it is lost or altered so that it cannot be recovered by subsequent alcohol precipitation. This confirms the findings of Katzman (10).

Efficacy of alcohol precipitation. Losses in dialysis. Since in urines of low gonado-

[2] Postmeuopausal urine activity (Gamone) concentrated by E. R. Squibb and Sons, New Brunswick, N. J.

tropic potency, the crude alcohol precipitate is too toxic to permit assay, dialysis was used in an attempt to reduce the toxicity. The dry alcohol precipitate was extracted three times with water, the combined aqueous extracts dialyzed in Viscose tubing overnight against running tap water and the activity then precipitated by the addition of 4 volumes of acetone and a trace of sodium acetate. This precipitate was suspended in saline and aliquots taken for injection. This procedure is the same as that used by Heller and Heller (4) but was developed independently. Our studies on the magnitude, cause, and prevention of losses in this process are of some interest. Table 2 shows the loss caused by the dialysis procedure as outlined above when it is applied to a pooled crude alcohol precipitate from postmenopausal urine. It is obvious from table 2 that the loss of 47 per cent of the activity may be due to extraction, dialysis, or acetone reprecipitation.

TABLE 2. LOSS OF ACTIVITY OF POSTMENOPAUSAL URINE DURING DIALYSIS

Aliquot and Treatment	M.U.U./day
A. Aqueous extract of crude ethanol precipitate dialyzed and reprecipitated with 4 volumes of acetone	200
B. Crude alcohol precipitate suspended in water	375

Table 3 shows the results of an attempt to localize the loss with respect to the three steps involved in the above treatment. The same pooled alcohol precipitate from postmenopausal urine was used. The data in this table indicate that the loss takes place entirely during dialysis. Attempts to decrease this loss by changing the conditions of dialysis were unsuccessful.

TABLE 3. LOSSES DUE TO THE THREE STEPS OF THE DIALYSIS PROCEDURE

Aliquot and Treatment	M.U.U./day
A. Alcohol precipitate suspended in water	450
B. Aqueous extract of alcohol precipitate	500
C. Dialyzed aqueous extract of alcohol precipitate	250
D. Dialyzed aqueous extract of alcohol precipitate reprecipitated with 4 volumes of acetone	250

A comparison of dialysis losses with the total activity present in a sample of postmenopausal urine is shown in table 4. In this experiment the entire sample of urine was extracted with n-butanol and ether to remove combined and free estrogens which interfere in the assay. The aqueous phase was treated as indicated. The yield by the alcohol method with dialysis is somewhat less than 50 per cent of the activity of the raw urine. That most of the loss is due to dialysis itself is shown by the fact that *aliquot B* which was dialyzed alone yields only 60 per cent of the raw urine's activity. This confirms the previous evidence that more than 75 per cent of

TABLE 4. COMPARISON OF ACTIVITY OF RAW POSTMENOPAUSAL URINE WITH CONCENTRATES PREPARED BY DIFFERENT METHODS

Aliquot and Treatment	M.U.U.
A. Precipitated with 4 vol. ethanol, extracted with water, dialyzed, reprecipitated with 4 vol. acetone	200–250
B. Untreated urine dialyzed	250–350
C. Untreated urine injected directly	450–550
D. Urine dialyzed and precipitated with 4 vol. ethanol	250
E. Activity precipitated with tannic acid; precipitate suspended and injected	toxic

the loss encountered in dialysis of the aqueous extract of the crude alcohol precipitate with subsequent precipitation by acetone is due to the dialysis itself.

Studies on the tannic acid precipitation of gonadotropin. The method as we used it was given to us by Dr. Levin in a personal communication, but has since been published in detail (13). Dr. Levin also sent us some tannate prepared by him from urine of normal males. This material when submitted to the alkaline decomposition recommended by him and assayed by us gave results which agreed very closely with his. This indicates that our alkaline treatment of the powder was efficient and did not cause loss of activity, and that our assay method gives results essentially the same as his. Since tannate precipitates were toxic in our hands, and since the tannic acid we used was not the same grade as that used by Levin, we injected the two tannic acids alone into mice and found that the lowest total dose used (0.5 mg.) spread over three days caused death in all the animals. This occurred somewhat sooner in the animals receiving our tannic acid, indicating that it was more toxic. When tannic acid is injected in combination with one M.U.U. of activity from postmenopausal urine, there is no lessening of tannic acid toxicity. Doses of 0.5 ,0.3, and 0.1 mg. of tannic acid per three days caused death in all animals at approximately the same time whether injected alone or mixed with the gonadotropin. This indicates that there is no lessening of toxicity in the mixture compared to the tannic acid alone as might be expected if a complex were formed between the two, thus lessening the amount of free tannic acid present to act as toxic agent.

TABLE 5. MOUSE UTERINE WEIGHT RESPONSE TO TANNIC ACID PRECIPITATE OF AQUEOUS EXTRACT OF CRUDE ALCOHOL PRECIPITATE COMPARED TO RESPONSE TO INJECTION OF UNTREATED EXTRACT

Treatment	Dose in mg. equivalents of alcohol powder	Average uterine weight
		mg.
Tannate precipitate of aqueous extract of alcohol preciptiate	13.8	5.1
	20.7	8.1
	26.7	24.6
Aqueous extract of alcohol precipitate	13.8	34.8
Control		4.1

In an effort to evaluate the completeness of precipitation of activity by the tannic acid method, two experiments were made. In the first an aqueous extract was prepared from an alcohol precipitate of postmenopausal urine and divided into two aliquots. One was injected without further treatment and the other was precipitated with tannic acid by the method of Levin including the alkaline decomposition of the tannate before injection. The results appear in table 5.

It is evident that under these conditions the tannate precipitate contained less than 50 per cent of the total activity present in the starting material. If raw postmenopausal urine after extraction with butanol and ether was used as the source of activity, the tannic acid precipitate contained less than 25 per cent of the activity present in the starting material.

Efficacy of the 50 per cent alcohol extraction of the crude alcohol precipitate to replace dialysis. Preliminary attempts to purify the crude alcohol precipitate from urine of normal males by the method of Gurin, Bachman, and Wilson (20) showed that there was a yield of more than 50 per cent of the activity with an 8.5-fold concentration of gonadotropin and a great reduction in the toxicity.

In view of the encouraging nature of these results it was decided to study the substitution of the 50 per cent ethanol extraction for the dialysis procedure on alcohol precipitates from normal urines. The crude alcohol precipitate from normal males was treated as follows.

Aliquot 1. Fifty per cent ethanol extract of the crude alcohol precipitate reprecipitated with two volumes of ethanol.

Aliquot 2. Aqueous extract of crude alcohol precipitate, dialyzed, and reprecipitated with 4 volumes of acetone.

Aliquot 3. Aqueous extract of crude alcohol precipitate, dialyzed and reprecipitated with 4 volumes of acetone. This precipitate was then extracted with 50 per cent ethanol and reprecipitated with 4 volumes of ethanol.

Aliquot 4. Fifty per cent ethanol extract of the crude alcohol precipitate, reprecipitated with 4 volumes of ethanol.

TABLE 6. RESPONSES TO PREPARATIONS MADE BY 50 PER CENT ETHANOL EXTRACTION OF CRUDE PRECIPITATE COMPARED TO THOSE MADE BY DIALYSIS

	Immature mouse uterine weight response to following dilutions				
	A	B	C	D	E
M.U.U. equivalents	0.50	0.75	1.0	1.5	2.0
Aliquot 1	6.4 (3)	7.0 (3)	14.7 (3)	10.2 (3)	10.3 (3)
Aliquot 2	6.7 (3)	8.3 (2)	10.9 (2)	Toxic	Toxic
Aliquot 3	Toxic	6.7 (3)	8.3 (2)	12.6 (4)	27.9 (2)
Aliquot 4	5.2 (4)	6.8 (4)	11.6 (4)	15.4 (4)	34.0 (2)

Four mice were used on each level. Numbers in parentheses indicate the animals surviving. 'Toxic' means that none of the mice survived.

TABLE 7. REPETITION OF WORK IN TABLE 6, USING MICE PER LEVEL

	Immature mouse uterine weight response to following dilutions				
	A	B	C	D	E
M.U.U. equivalents	0.50	0.75	1.0	1.5	2.0
Aliquot 1	7.8 (8)	9.5 (8)	18.7 (8)	30.3 (8)	32.1 (8)
Aliquot 2	7.1 (8)	6.8 (3)	14.0 (3)	34.0 (1)	Toxic
Aliquot 3	7.2 (7)	11.1 (7)	22.6 (3)	36.7 (2)	19.5 (5)
Aliquot 4	7.1 (8)	11.5 (8)	25.9 (8)	34.5 (8)	30.0 (5)

Eight mice were used on each level. Numbers in parentheses indicate the animals surviving. 'Toxic' means that none of the mice survived.

In the alcohol extraction process, since the pH of the 50 per cent alcohol mixture was difficult to measure with the glass electrode, the adjustment was made by means of external indicators to pH 5.4 to 6.8. The results are shown in table 6. Inspection of the table shows that the alcohol method is better than the dialysis method from the standpoint of recovery as well as from that of reduction of toxicity. Combination of the routine water extraction, dialysis and acetone precipitation followed by 50 per cent ethanol extraction and reprecipitation with 4 volumes of alcohol (*aliquot 3*) gives a better recovery than the water extraction and dialysis alone (*aliquot 2*) but not as good as the 50 per cent alcohol extraction alone (*aliquots 1* and *4*). Repetition of this experiment using 8 mice on each level and keeping the volumes as low as possible gave the results shown in table 7. The data show that recovery by the 50 per cent alcohol extraction followed by precipitation with 4 volumes of alcohol (*aliquot 4*) is better than by any other method. Next best is the combination of routine water extraction followed by 50 per cent alcohol extraction of the acetone precipitate from the dialyzed water extract (*aliquot 3*).

Confirmatory results are given in table 8 in which we show the high toxicity of the 50 per cent alcohol insoluble fraction and of the crude alcohol precipitate from urine of normal males. The aliquots recorded in table 8 were prepared as follows.

Aliquot 1. Fifty per cent ethanol extract of crude preparation reprecipitated by 4 volumes of ethanol.

Aliquot 2. Residue from *aliquot 1* (not soluble in 50 per cent ethanol) extracted with water and precipitated with 4 volumes of acetone.

Aliquot 3. Aqueous extract of crude alcohol precipitate dialyzed and precipitated with 4 volumes of acetone.

Aliquot 4. Aqueous extract of crude alcohol precipitate dialyzed and precipitated with 4 volumes of acetone. This precipitate was then extracted with 50 per cent ethanol and reprecipitated by 4 volumes of ethanol.

Aliquot 5. Original crude alcohol precipitate suspended, untreated.

In table 8 comparison of *aliquots 1*, *2*, and *5* shows an excellent separation of the toxic and gonadotropic factors. *Aliquot 1* compared to *aliquot 3* at the 1.0 and 0.75

TABLE 8. RESPONSE TO 50 PER CENT ALCOHOL EXTRACTS COMPARED TO AQUEOUS EXTRACTS AND TO RESIDUE AFTER ALCOHOL EXTRACTION

	Immature mouse uterine weight response to following dilutions			
M.U.U. equivalents	A 0.75	B 1.0	C 1.5	D 2.0
Aliquot 1	13.2 (7)	16.1 (6)	21.8 (7)	22.9 (7)
Aliquot 2	6.7 (3)	Toxic	Toxic	Toxic
Aliquot 3	10.1 (7)	12.2 (8)	21.3 (5)	21.9 (2)
Aliquot 4	8.4 (7)	17.5 (7)	23.0 (8)	27.1 (8)
Aliquot 5	Toxic	Toxic	Toxic	Toxic

Eight mice were started on each level. Numbers in parentheses indicate the animals surviving. 'Toxic' means that none of the mice survived.

M.U.U. levels shows that the final product of the 50 per cent ethanol extraction (*aliquot 1*) contains about one-third more activity than was obtained by water extraction followed by dialysis (*aliquot 3*). From results on *aliquots 1* and *4* we conclude that the alcohol extraction of the acetone precipitate of the aqueous extract subsequent to dialysis causes no appreciable additional loss in activity, but a marked lowering of toxicity. This observation is good confirmatory evidence for the completeness of the alcohol extraction and also clearly shows that removal of toxicity is more efficient by the 50 per cent alcohol extraction than by dialysis, since even after dialysis additional toxic material is removed by the alcohol extraction procedure.

Another experiment designed to characterize the toxic fraction of urine from normal males from the active material confirms the value of the 50 per cent alcohol procedure. Starting from the crude alcohol precipitate (*fraction 1*) four additional fractions were prepared as follows.

Fraction 2. Fifty per cent ethanol extract of crude alcohol precipitate reprecipitated with 4 volumes of alcohol.

Fraction 3. Aqueous extract of the 50 per cent alcohol insoluble residues from *fraction 2*, finally precipitated by four volumes of ethanol.

Fraction 4. Residue remaining after water extraction in *fraction 3*.

Fraction 5. An aliquot of *fraction 3* re-extracted with water and reprecipitated by making the concentration of alcohol 50 per cent.

Fraction 2 on the basis of total solids per mouse uterine unit was found to represent a fivefold concentration of the gonadotropic activity and a lowering in toxicity

to one-fourth that of the original powder. This purification was accomplished with a yield of more than 65 per cent of the gonadotropic activity. *Fraction* 3 exhibits the same toxicity on a unit equivalent basis as the unfractionated extract, but there was a 1.6-fold concentration of the toxicity on a weight basis. *Fraction* 4 was more toxic than the starting powder or than *fraction* 3 on a weight basis, but not as toxic as either of them on a unit equivalent basis. *Fraction* 5 represented as much toxicity on a weight basis as the original powder and was very low in, or free from gonadotropic activity.

Thus it was shown that the toxicity and gonadotropic activity can be separated into two well-defined fractions by the 50 per cent ethanol extraction.

Two recovery experiments on the crude alcohol precipitate from postmenopausal urines were carried out because the activity per unit weight is so high that the toxicity factor can be eliminated. Thus a direct comparison of the 50 per cent ethanol extraction followed by reprecipitation with 4 volumes of alcohol, and of the water extrac-

TABLE 9. VOLUME EFFECT

	Mouse uterine weight response to following dilutions			
	A	B	C	D
M.U.U. equivalents	0.75	1.0	1.5	2.0
Mg. equivalents	5.25	7.0	10.5	14.0
Aliquot 1	6.5 (8)	9.9 (8)	23.6 (8)	23.3 (8)
Aliquot 2	7.4 (8)	11.9 (7)	23.2 (8)	26.5 (8)
Aliquot 3	6.4 (8)	9.9 (8)	22.4 (7)	28.4 (8)
Aliquot 4	7.5 (6)	8.0 (8)	22.4 (7)	21.4 (8)
Aliquot 5	7.6 (8)	20.2 (8)	22.6 (8)	28.1 (8)
Aliquot 6	7.4 (8)	14.1 (8)	27.5 (8)	29.2 (8)

Eight mice were started on each level. Numbers in parentheses indicate the animals surviving.

tion and dialysis followed by reprecipitation with 4 volumes of acetone, can be made with the original alcohol precipitate. The results from the first of these experiments showed that the 50 per cent alcohol extraction and the dialysis procedures gave recoveries of 75 and 60 per cent respectively. In the second of these experiments an attempt was made to test the effect of changes in the volume of the 50 per cent ethanol used to extract the crude alcohol precipitate of postmenopausal urine. In table 9 *aliquots* 1 and 2 were extracted with 11.5 cc. of 50 per cent ethanol each; *aliquot* 1 was precipitated with 4 volumes and *aliquot* 2 with 8 volumes of alcohol. *Aliquots* 3 and 4 were extracted with 30 cc. of 50 per cent ethanol each; *aliquot* 3 was precipitated by 4 volumes and *aliquot* 4 by 8 volumes of alcohol. *Aliquot* 5 was a suspension of the crude powder injected without further treatment, and *aliquot* 6 was an aqueous extract of the crude powder precipitated by the addition of 8 volumes of alcohol.

Obviously a two-fold dilution in 50 per cent ethanol used for extraction does not affect the yield of gonadotropin, and precipitation by 8 volumes of ethanol gives essentially the same results as precipitation by 4 volumes. Comparison of the yields by the alcohol extraction method with those in the last two aliquots again shows that the yield by the 50 per cent alcohol method is slightly less than 100 per cent of the total activity whether the latter be based on the reprecipitation of the aqueous extract (*aliquot* 6) or on injection of the suspended powder (*aliquot* 5). The results also suggest that the water extract (*aliquot* 6) does not give as high a response as the suspension of the crude alcohol precipitate. Whether the slight difference was due to incomplete extraction and/or reprecipitation, or whether injection of the suspension

leads to a more gradual release of the gonadotropin because of temporary adsorption of the activity on inert material was not determined.

In table 10 are given the results of an experiment in which the toxic and nontoxic fractions obtained by the 50 per cent alcohol-extraction procedure were recombined as indicated. It is obvious that the toxic fraction in the doses used does not lower the gonadotropin response in the immature mouse. The results suggest instead a possible augmentation. A similar possible augmentation was observed in the toxic fraction

TABLE 10. RECOMBINATION OF GONADOTROPIC AND TOXIC FRACTIONS FROM POSTMENOPAUSAL URINE

Mouse uterine unit equivalents of toxic fraction	Mouse uterine unit equivalents of non-toxic fraction		
	0.75	1.0	1.5
	mg.	mg.	mg.
0	5.1	12.7	17.3
1	5.7	10.2	23.4
2	5.5	15.2	34.0
4	9.9	15.3	26.5

The figures represent the average uterine weight response to combinations of the two fractions expressed as mouse uterine unit equivalents.

from the urine of normal men. It is probable that a very low content of gonadotropin in the toxic fraction is not the cause of the slight augmentation because no gonadotropin activity was detected in the toxic fraction although the equivalent of 6 M.U.U. was injected. However, it is possible that the undetected activity, if present, may increase the response when it is combined with the nontoxic gonadotropin fraction, or that the toxic fraction decreases the rate of absorption and thus causes an augmentation. It is obvious that recovery studies on urinary gonadotropin from normal urine may be particularly involved due to these complications.

Effect of alkaline treatment on activity of alcohol precipitate. Since Levin uses alkali to decompose the tannate and to extract the alcohol precipitate in his modification of this procedure, we tested the effect of alkaline extraction on our 50 per cent alcohol procedures. Five aliquots of the crude alcohol precipitate of pooled urine from normal males were treated as follows.

Aliquot 1. Extracted with water, dialyzed, and precipitated with 4 volumes of acetone plus a trace of sodium acetate.

Aliquot 2. Extracted with dilute alkali (pH 8.5), dialyzed and precipitated with 10 volumes of acetone plus a trace of sodium acetate.

Aliquot 3. Extracted with 50 per cent ethanol (pH 6.3), not dialyzed. Precipitated with 4 volumes of alcohol plus a trace of sodium acetate.

TABLE 11. EFFECT OF ALKALI TREATMENT

	Mouse uterine weight response to following dilutions				
M.U.U. equivalents	A 0.75	B 1.0	C 1.25	D 1.5	E 2.0
Aliquot 1	10.4 (2)	8.5 (2)	Toxic	24.5 (3)	Toxic
Aliquot 2	7.4 (3)	8.8 (1)	18.2 (3)	8.8 (1)	25.7 (4)
Aliquot 3	8.5 (4)	23.2 (3)	24.4 (4)	26.5 (3)	21.7 (4)
Aliquot 4	21.1 (4)	15.0 (3)	13.5 (4)	29.5 (4)	21.5 (1)
Aliquot 5	7.9 (4)	21.1 (4)	16.3 (4)	25.8 (4)	28.7 (4)

Four mice were started on each level. Numbers in parentheses indicate the animals surviving. 'Toxic' means that none of the mice survived.

Aliquot 4. Extracted with 50 per cent ethanol (pH 6.3), dialyzed, and precipitated with 4 volumes of alcohol plus a trace of sodium acetate.

Aliquot 5. Extracted with 50 per cent ethanol at pH 8.5, dialyzed, and reprecipitated with 4 volumes of ethanol plus a trace of sodium acetate.

The responses to these solutions are given in table 11. The 50 per cent alcohol method (*aliquot 3*) gave the best yield and the alkaline alcohol extraction followed by dialysis (*aliquot 5*) was next best, being better than the alcohol extraction at pH 6.3 (*aliquot 4*) which was also dialyzed. Due to toxicity it is impossible to detect any difference between the aqueous extracts with and without alkali, but the 50 per cent alcohol extraction again resulted in a better yield than when the water extraction followed by dialysis was used. We conclude that the alkaline extraction does not decrease the yield per se, but that it is undesirable in conjunction with the 50 per cent ethanol extraction method because its use makes it necessary to employ dialysis which does decrease the yield.

SUMMARY

By the immature mouse uterine weight response assay method for gonadotropin it was found that:

1. The precipitation of urinary gonadotropic activity by the addition of 4 volumes of alcohol gives higher yields than the tannic acid or the benzoic acid method, but the product from normal human urine is too toxic to permit direct injection.

2. Aqueous extraction of the crude alcohol precipitate followed by dialysis and reprecipitation causes a loss of activity due mainly to the dialysis rather than to the extraction or reprecipitation.

3. Extraction of the crude alcohol precipitate with 50 per cent ethanol and reprecipitation of the activity from the solution by the addition of 4 volumes of alcohol is a more efficient purification method than dialysis from the standpoints of yield of activity and reduction of toxicity.

4. The toxic material which is insoluble in 50 per cent ethanol has no inhibiting effect on urinary gonadotropic activity when the two fractions are mixed and injected.

5. Attempts to purify the toxic material were not successful.

6. Use of weakly alkaline 50 per cent ethanol as the extraction agent did not alter the yield of activity.

REFERENCES

1. Zondek, B.: 1st ed. Die Hormone des Ovariums und des Hypophysenvorderlappens. Julius Springer, Berlin, 1931.
2. Albright, F., J. A. Halsted and E. Cloney: *New England J. Med.* 212: 192. 1935.
3. Saethre, H.: *Klin. Wchnschr.* 12: 1409. 1933.
4. Heller, C. G., and E. J. Heller: *Endocrinology* 24: 319. 1939.
5. McCullagh, D. R., and W. E. Bowman: *Endocrinology* 27: 525. 1940.
6. Katzman, P. A., and E. A. Doisy: *Proc. Soc. Exper. Biol. & Med.* 30: 1188. 1933.
7. Katzman, P. A., and E. A. Doisy: *Proc. Soc. Exper. Biol. & Med.* 31: 188. 1933.
8. Katzman, P. A., and E. A. Doisy: *J. Biol. Chem.* 106: 125. 1934.
9. Katzman, P. A., and E. A. Doisy: *J. Biol. Chem.* 98: 739. 1932.
10. Katzman, P. A.: *Endocrinology* 21: 89. 1937.
11. Levin, L., and H. H. Tyndale: *Proc. Soc. Exper. Biol. & Med.* 34: 516. 1936.
12. Levin, L., and H. H. Tyndale: *Endocrinology* 21: 619. 1937.
13. Levin, L.: *Endocrinology* 28: 378. 1941.
14. Friedman, M. H. and G. L. Weinstein: *Endocrinology* 21: 489. 1937.
15. von Haam, E., and N. O. Rothermich: *Proc. Soc. Exper. Biol. & Med.* 44: 369. 1940.
16. Leonard, S. L., and P. E. Smith: *Am. J. Physiol.* 108: 22. 1934.
17. Evans, J. S., L. Hines, R. Varney and F. C. Koch: *Endocrinology* 26: 1005. 1940.
18. Evans, J. S., R. F. Varney and F. C. Koch: *Endocrinology* 28: 747. 1941.
19. Heller, C. G., H. Lauson and E. L. Sevringhaus: *Am. J. Physiol.* 121: 364. 1938.
20. Gurin, S., C. Bachman and D. W. Wilson: *J. Biol. Chem.* 128: 525. 1939.

ELIMINATION OF THE PARS NERVOSA[1] WITHOUT ELICITING DIABETES INSIPIDUS[2]

ALLEN D. KELLER

From the Department of Physiology and Pharmacology
University of Alabama School of Medicine
TUSCALOOSA, ALABAMA

IT IS WELL ESTABLISHED (5–9) that permanent diabetes insipidus can be precipitated by a bilateral, destructive lesion placed in the anterior ventral hypothalamus (tuberal middle region or supraoptic middle region). Because such a lesion interrupts a prominent component (supraoptico-hypophysial tract) of the infundibular bundle of nerve fibers it has been assumed that intact hypothalamico-hypophysial fibers are essential for the continued maintenance of a normal fluid exchange (denervation theory (7, 8, 10). The champions of the denervation theory in its most recent form, in addition, contend that the antidiuretic-elaborating tissue is rigidly confined to the pars nervosa ofithe hypophysis; and stress that the infundibulum possesses potent antidiuretic elaborating powers in common with the infundibular process (11–15). The recent anatomical studies of Gersh (16), in spite of the fact that his physiological evidence is entirely circumstantial in nature, have been widely accepted as proof of the correctness of both of these contentions.

In a previous report (17) evidence was presented which cast grave doubt upon the acceptance of the denervation theory as it is delimited above. In this paper are demonstrated the selective destruction of the pars nervosa in the cat, and the attainment of total hypophysectomy in the dog without precipitating diabetes insipidus (4, 19). These experiments verify our earlier results and conclusions and further indicate that tissue other than that of the pars nervosa possesses the power of elaborating the antidiuretic principle.

EXPERIMENTAL PROCEDURES

Operative procedures. Cats. In the experiments on cats the ventral extent of the hypothalamus (ventral portion of tuberal middle region) was isolated from the rest of the hypothalamus by one sweep of a long narrow blunt probe, after which the isolated tuberal tissue was removed with forceps. Such isolated hypothalamic tissue usually pulls free from the hypophysial stalk with ease leaving it relatively undisturbed insofar as direct operative visibility reveals. The space left vacant by the removal of the hypothalamic tissue was plugged by bits

Received for publication December 29, 1941.

[1] Pars nervosa is used as synonymous to Wislocki and King's (1) and Rioch, Wislocki and O'Leary's (2) usage of neurohypophysis. Pars nervosa is used in preference to neurohypophysis because as this paper indicates it seems likely that the term neurohypophysis should be extended to include a hypothalamic component in addition to the pars nervosa proper.

The pars nervosa is subdivided into the *infundibular process* (enlarged distal component of the posterior lobe) and the *infundibulum* (stalk component) (2, 3). For purposes of simple description the infundibulum is merely subdivided into its distal and proximal portions. The median eminence comprises the most proximal extent of the infundibulum. The term infundibular is used throughout the paper with reference to the infundibulum or infundibular process. In referring to the ventral middle region of the hypothalamus, tuberal is used instead of infundibular in order to avoid possible confusion.

[2] Aided by a grant from the Rockefeller Foundation. Read before the American Physiological Society in Chicago, April 18, 1941 (4).

of muscle taken from the cut surface of the temporal muscle (see sections *B* and *C*, fig. 2). With due care the completeness of the isolation of the hypophysis can be readily verified with certainty at operation. The whole procedure is literally bloodless unless surface arteries are pulled. When this occurs, bleeding is usually easily controlled by repeated application of cotton pledgets.

This procedure routinely assures the complete separation of all hypophysial tissue from the hypothalamus, and in addition, frequently results in the subsequent disappearance of all but a fragmentary peripheral rim of pars nervosa tissue (see sections *A* to *G* in fig. 2).

Dogs. The hypophysectomy procedures on dogs were carried out by exposing the hypophysis and ventral hypothalamus on the right side by elevation and retraction of the temporal pole and section of the oculomotor nerve. This exposure routinely gives excellent visualization of the distal portion of the hypophysial stalk and frequently the entire stalk. In performing an ordinary hypophysectomy the stalk was cut proximal to the anterior tip of the pars anterior. Subsequent to stalk section, the gland tissue distal to the section was removed by manipulation with forceps. The tissue of the stalk proximal to the section remained, with normal vascular supply, attached to the hypothalamus (see sections *A* to *D*, fig. 4). Following the removal of the gland, the depth of the pituitary fossa was either cauterized with an electric cautery or swabbed with a cotton pledget soaked in a concentrated phenol solution.

Attempts to execute a total hypophysectomy (complete removal of the entire pars nervosa and adenohypophysis) without infringement upon the hypothalamus were made only when the entire hypophysial stalk was readily visible. The stalk was then cut across at its junction with the hypothalamus (see sections *E* to *H*, fig. 4). When fragments of pars tuberalis remained attached to the hypothalamus they were clearly visible because of their characteristic greyish appearance and were subsequently carefully cauterized but often at the expense of encroachment upon hypothalamic tissue (see sections *I* to *L*, fig. 4).

In the experiments in which drastic infringement upon the hypothalamus plus total hypophysectomy (see *I* to *L*, fig. 5) was desired the ventral hypothalamic tissue was first isolated by manipulation with a blunt probe, following which hypophysectomy was executed with subsequent cauterization of the junctional hypothalamic-hypophysial stalk tissue.

Animal regime. Cats. Adult cats in good physical condition and previously adapted to our cage regime were used. The weights ranged from 2 to 3 kg. They were maintained on one of two diets or alternately on the two. One diet consisted of 100 cc. of undiluted evaporated milk and 50 gm. of Baloration baited with salmon or 50 gm. of beef stew meat. The second diet consisted of 50 cc. of undiluted evaporated milk and 100 gm. of meat or 100 gm. of Baloration baited with salmon. This procedure differed from our previous routine in that the foot intake was maintained constant in an attempt to stabilize the salt intake and accordingly the water exchange. As will be pointed out later, we found the shift of milk intake from 100 cc. to 50 cc. and vice versa an added tool to segregate cases of questionable diabetes insipidus. The amount of milk intake was routinely included in charting fluid consumption.

Dogs. Adult female dogs in good physical condition ranging in weight from 5 to 9 kg. were used. They were routinely maintained on Baloration, 50 gm. per kg. of body weight, but not over a total of 300 gm. per day. Subsequent to operation, the preoperative daily meal was divided into 12-hour feeding intervals. In addition, for the first 21 to 28 postoperative days the dogs were given some sugar in their drinking water.

Cats and dogs. When thyroid was administered one gram[3] was given daily in the food or by capsule for a 6 to 10-day period. Anterior lobe extract[3] was administered subcutaneously, 0.5 cc. daily over a similar period. One mg. of synthetic thyroxin[3] was given intravenously daily for a 5-day period.

Water was available to the animals for 22 hours of each 24. The urine output and urine specific gravity were not recorded routinely but at intervals, continuous recording being impracticable due to inadequate cage facilities.

Histological procedures. The animals were killed with ether, and the brain was perfused

[3] Thyroid as U.S.P. powder was supplied by Eli Lilly & Company, Indianapolis Indiana; the anterior pituitary extract was the "total extract" of Parke, Davis & Co., Detroit, Michigan; synthetic thyroxin was supplied by Roche-Organon of Nutley, New Jersey.

with 10 per cent formalin through the heart. After 48 hours of hardening in 10 per cent formalin the hypothalamico-hypophysial block was sectioned, either across or sagittally, at 10, 15 or 20μ. Three sets of sections consisting of every 10th or 20th section were routinely mounted and stained, a different stain being employed for each set. The first set was stained

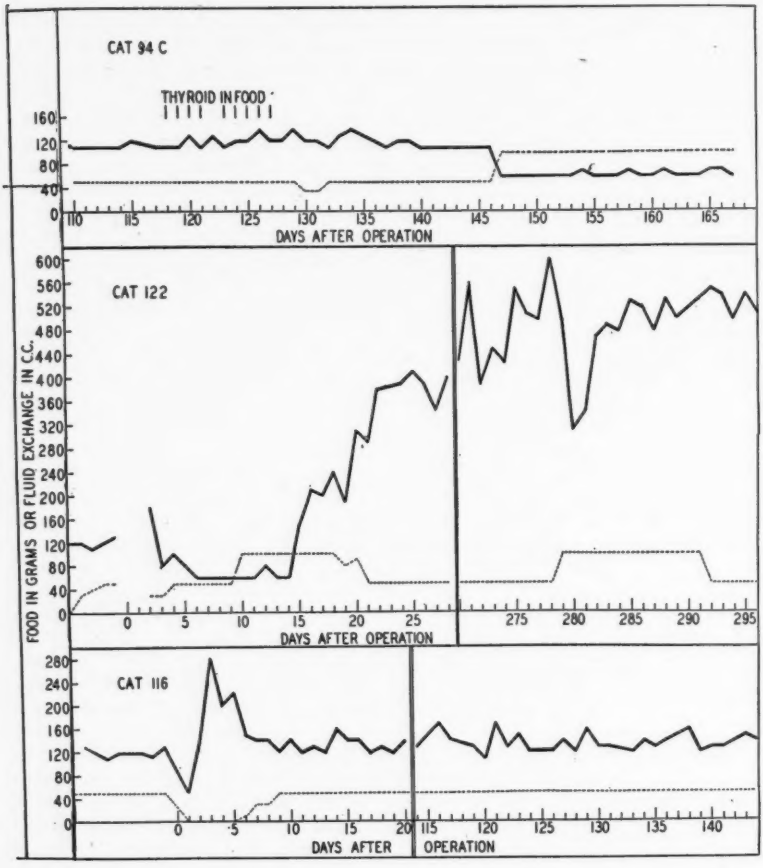


Fig. 1. TOTAL DAILY FOOD AND FLUID INTAKE CURVES FOR CATS AS LABELLED. Fluid intake is indicated by the solid lines and food intake by the broken lines. Note: *a*) there was failure of thyroid administration to elicit any appreciable increase in the fluid intake in *cat* 94C. *b*) there was an abrupt, permanent drop in fluid intake in *cat* 94C when the milk intake was reduced and replaced by additional solid food in the diet (146th day) while the same procedure in *cat 122* (278th day) resulted in only a very transitory drop in fluid intake. *c*), there was a close approximation of the preoperative and postoperative fluid intake in *cat 116* (no residual diabetes insipidus) as compared with the marked postoperative rise which remained permanent in *cat 122*.

to show medullated fiber tracts (modified Pal-Weigert). The second set consisting of the sections immediately adjacent to those of the first set was stained with cresyl violet. The third set was made up of the sections immediately consecutive to the sections of the second set and was stained either by a modified Maximow or Mallory stain. Subsequent to these routine procedures sections were mounted for special staining such as for Bodian's.

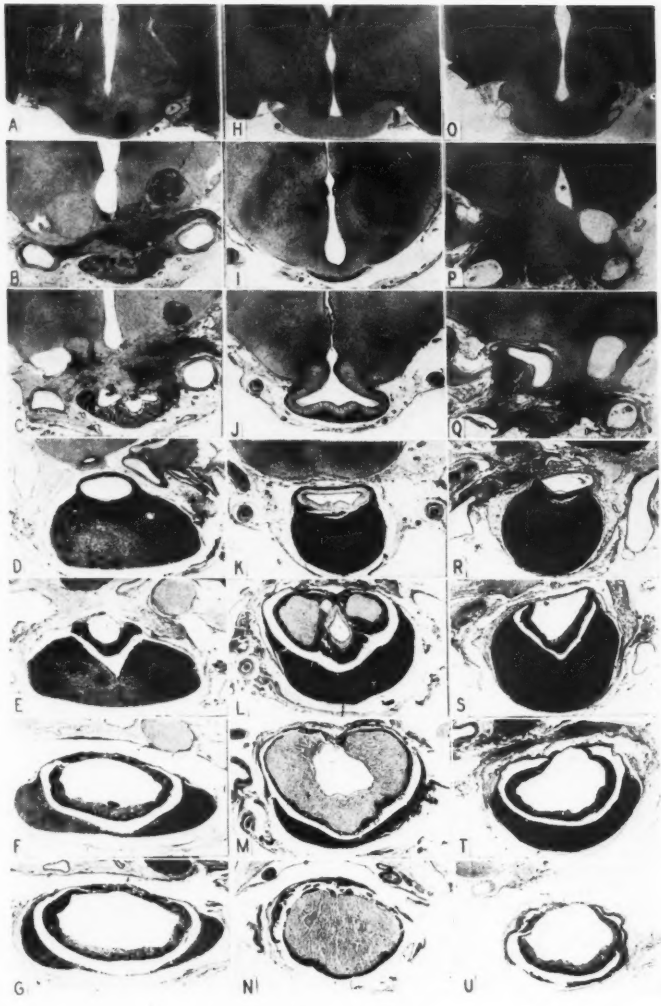


Fig. 2. LOW POWER PHOTOMICROGRAPHS OF REPRESENTATIVE SECTIONS OF HYPOTHALAMIC AREAS taken from the cresyl violet serials of a normal cat (central vertical column) *cat 116* (left vertical column) and *cat 122* (right vertical column). The sections in each horizontal row are cut at approximately the same level and accordingly can be compared one with the other. Note particularly: *a*), There is a total absence of the supraoptic nuclei, unassociated with glial proliferation, in *cat 122* (*section O*) while these nuclei in *cat 116* show no noticeable deviation from the normal; compare *sections A*, and *H*. *b*) There is complete demarcation of the entire hypophysis from the hypothalamus by the lesion scars, which in turn involve the ventral extent of the tuberal middle region of the hypothalamus, in both *cats 116* and *122* (compare *sections B* and C with *I* and *J*, and *P* and *Q* with *I* and *J*). *c*), There is essentially a total absence of the tissue of the pars nervosa with a corresponding enlargement of the infundibular cavity in both *cats 116* and *122* (compare *sections B* to *G* with *I* to *N* and *P* to *U* with *I* to *N*). d), In both of these cats the adenohypophysis remains undisturbed.

RESULTS

Cats. There were 8 cats in this particular group of experiments. Four of the animals exhibited no deviation from the normal in their fluid exchange nor any latent tendencies toward diabetes insipidus as tested by thyroid feeding, or a change from 100 cc. to 50 cc. of milk or vice versa. Three of the animals showed no change from the normal in their routine daily water exchange subsequent to operation, but did give some evidence of latent diabetic tendencies. Only one animal of the eight developed a definite permanent diabetes insipidus. *Cats 116* and *94C* from this group are described and illustrated below. *Cat 122* is from another group of experiments and is described and illustrated for comparative purposes.

Histological studies in every instance verified complete separation of the hypophysis from the hypothalamus at the expense of destruction (actual removal) of considerable tuberal tissue (see sections *A* to *G*, fig. 2) and also disclosed a greater in-

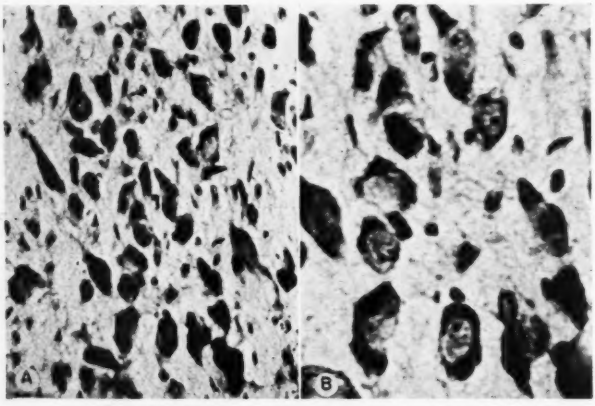


Fig. 3. *A.* Low power photomicrograph of a portion of the left supraoptic nucleus of *cat 116* (see *section* A, fig. 2). *B.* A high power photomicrograph of some of the cells shown in *A*.

fringement upon the tissue of the pars nervosa (central disappearance of tissue resulting in enlargement of the infundibular cavity) than was evident in our original experiments in which the hypothalamic tissue defect was not plugged with muscle (17, 18).

An instance of the selective destruction of essentially the entire pars nervosa without a subsequent residual diabetes insipidus is illustrated for *cat 116* in figures 1 and 2. It is most unlikely that secretory elements remained functional in the narrow peripheral rim of pars nervosa tissue that remained lining the enlarged infundibular cavity. But granting that such might be the case, the completeness of the separation of the entire hypophysis, including the recently much discussed median eminence (proximal infundibulum), from direct hypothalamic connections can not be questioned. In addition, the tissue of this cat (*116*) illustrates that the cells of the supraoptic nuclei do not invariably degenerate following severance of the infundibular nerve fibers as is commonly believed. This particular cat was autopsied 9½ months after operation, yet there was no drastic disappearance of cells from these nuclei as is evident in contrasting *A* with *H* in figure 2. The high-power photomicrographs shown in figure 3 demonstrate that there were numerous well defined cells in these nuclei.

A striking, persistent diabetes insipidus may, however, follow the separation of the hypophysis from the hypothalamus by our operative procedure. This is demon-

strated by *cat 122*. The food and fluid intake curves and the tissue photographs for this cat are likewise illustrated in figures 1 and 2. It was attempted at operation to separate, in this cat, the gland from the hypothalamus at their junction. It will be noted from the sections that although a complete separation was attained, there was also considerable infringement upon the ventral hypothalamic tissue. The gross

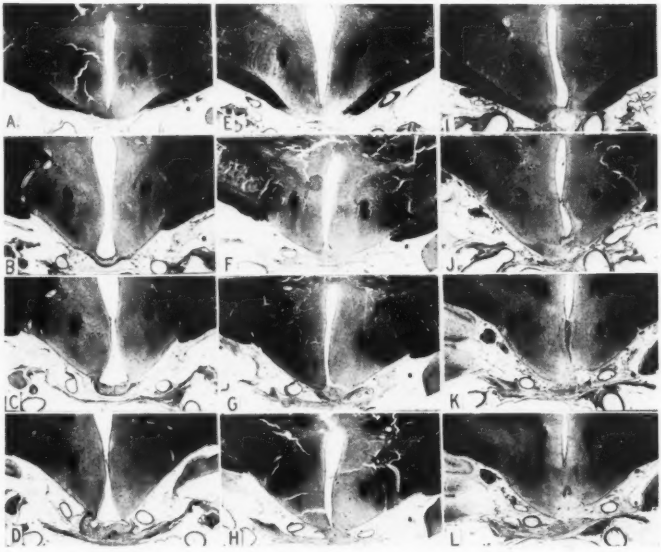


Fig. 4. LOW POWER PHOTOMICROGRAPHS OF REPRESENTATIVE SECTIONS OF HYPOTHALAMUS taken from sets stained by the Pal-Weigert method and counterstained with cresyl violet.

The sections in the left vertical column are from the set for *dog 53 Br* and visualize the tissue that remains intact following an ordinary hypophysectomy. In *A* the hypothalamus is cut through just caudad to the optic chiasm and just cephalad to the junction of the infundibulum with the hypothalamus. In *B* the hypothalamus is cut through just caudal to the cephalic extent of the infundibulum, the latter being surrounded ventrally and laterally by the cephalic strip of pars tuberalis. Note particularly the clear-cut demarcation of the infundibulum (median eminence) from hypothalamic tissue. In sections *C* and *D* the demarcation between these two tissues is even more pronounced.

The sections in the middle vertical column are from the set for *dog 290C* and illustrate an anatomically uncomplicated total hypophysectomy. Section *E* is cut at the same level as *A*. Section *F* is cut at the same level as *B*. Note that in both these sections, *E* and *F*, the hypothalamus is not disturbed and that no infundibulum tissue remains in *F*. In the place normally occupied by this tissue the ventral extent of the third ventricle is plugged or capped by a nodule of organized muscle and necrotic infundibular tissue. In sections *G* and *H* the nodule of organized tissue is more pronounced while it is evident there was no infringement upon hypothalamic tissue.

The sections in the right vertical column are from the series for *dog 15 Br* and illustrate a total hypophysectomy plus a mild infringement upon the hypothalamus. Note that the hypothalamic involvement extends from the caudal aspect of the chiasm cephalad to the mammillary bodies caudad.

tuberal tissue defect, however, was not as extensive as that in *cat 116*. In comparing section *O* with sections *H* and *A* (fig. 2) it will be noted that our operative procedure does at times cause degeneration of the supraoptic nuclei. In this particular instance absolutely all of the cells were gone whereas usually some cells remain, even when marked degeneration occurs. The central sloughing of the entire pars nervosa was entirely equivalent in *cats 116* and *122* (compare sections *B* to *G* with sections *P* to *U*, in fig. 2).

There are two minor observations that hold in cats having permanent diabetes insipidus which are illustrated in the graphs for *cat 122*. First, during a 5 months' lapse

of time between the first and second parts of the record, this cat's fluid exchange increased. This increase is explainable on the basis of a considerable increase in weight (from kitten to adult) of from 1.5 to 2.6 kg., thus illustrating that the magnitude of the fluid exchange, for a given deficiency in the antidiuretic factor, is closely associated with body weight (19). Secondly, when the cat's daily milk intake was reduced from 100 cc. to 50 cc. and the solid food intake increased from 50 to 100 gm.,

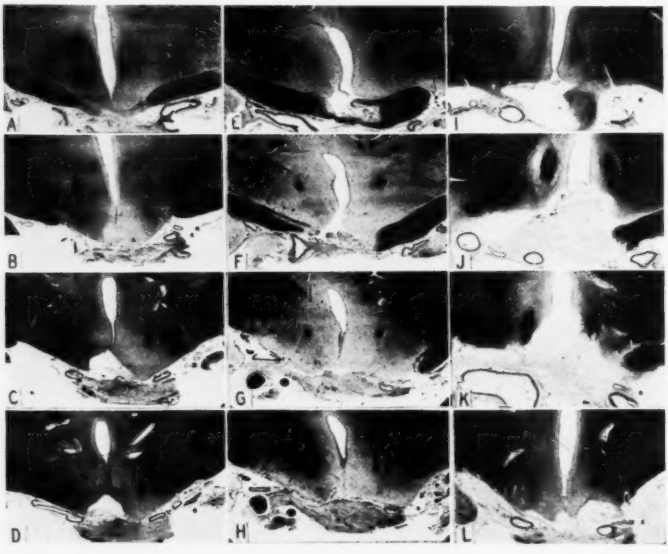


Fig. 5. LOW POWER PHOTOMICROGRAPHS OF REPRESENTATIVE SECTIONS OF HYPOTHALAMUS TAKEN from sets stained by the Pal-Weigert method and counterstained with cresyl violet (compare with sections in fig. 4).

The sections in the left vertical column are from the set for *dog 16 Br* and illustrate a total hypophysectomy plus a mild infringement upon the hypothalamus. Note that this infringement extends from the tissue immediately caudal to the optic chiasm anteriorly to the mammillary bodies posteriorly.

The middle vertical column of sections are from the set for *dog 13 Br* and illustrate a greater gross hypothalamic tissue defect than in *dog 16 Br*, by reaching further dorsally and actually involving the posterior portion of the optic chiasm anteriorly.

The right vertical column of sections are from the set for *dog 5 Br* and illustrates a still greater gross hypothalamic tissue defect than in *13 Br*. Note that the tissue defect reaches dorsally to the fornix and anteriorly through the whole of the optic chiasm (anterior hypothalamus).

the total fluid intake suffered only a temporary decrease, demonstrating the constancy of the level of the increased fluid exchange regardless of the inorganic salts in the diet. The effect is quite different for the nondiabetic cat as is shown in the curve for *cat* 94C in figure 1.

The fluid and food intake curves for *cat* 94C are illustrated at the top of figure 2. This cat was purchased when it was only half grown and allowed to run free about the medical building and the University environs. When the cat was full grown, the ventral extent of the hypothalamus was removed as described under operative procedures. Following operation the cat was returned immediately to its unrestricted routine. Three months after operation, when the cat was in excellent physical condition, it was caged and the fluid intake determined as is illustrated in figure 1. The fluid intake did not deviate from the normal nor was it affected materially by thyroid feeding. At termination complete severance of the hypophysis with marked en-

croachment upon the pars nervosa was verified by serial sections. The cells in the supraoptic nuclei were considerably reduced in number yet many well defined cells remained.

Dogs. Our results on dogs are illustrated by representative animals selected from a large and extended series of experiments centered about various hypophysectomy procedures over a period of several years.

Section of the hypophysial stalk through its middle (*dog 53 Br, A* to *D* fig. 4), with or without removal of the isolated gland, can be done without precipitating a permanent residual diabetes insipidus (21, 22). Following such a procedure a temporary

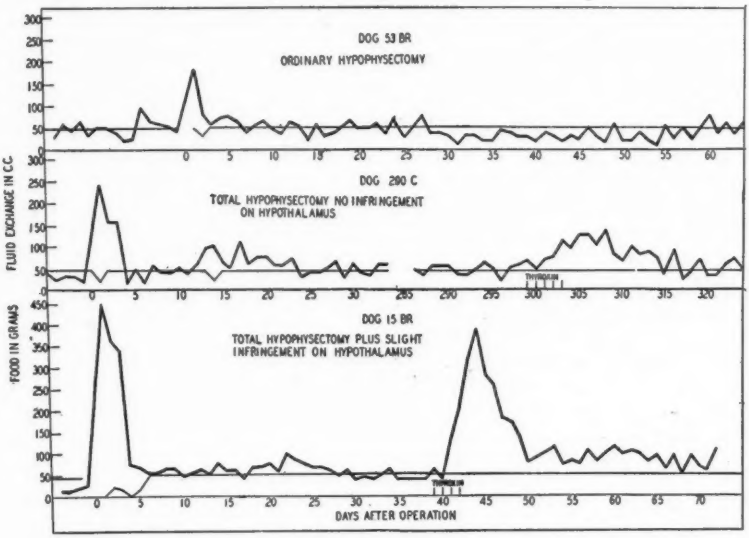


Fig. 6. Food and fluid intake curves for dogs indicated calculated per kg. of body weight on the basis of operative weight. Fluid intake in each instance is graphed by the heavy line, and the food intake by the light line. Thyroxin, 1.0 mg., was given intravenously on the days indicated. Note the absence of residual diabetes insipidus in all three dogs. Also that *dog* 290C exhibited only a perceptible tendency toward a latent diabetes insipidus while there was no question of a latent diabetes insipidus being present in *15 Br.*

diabetes insipidus may or may not occur, as was early observed and clearly described and illustrated by Cushing (23). Such animals in the chronic state, likewise, exhibit no latent diabetes insipidus as tested by thyroid, anterior lobe extract or thyroxin administration.

When the stalk is sectioned through its most proximal extent with subsequent removal of the isolated gland, there is considerable variation in the way in which it affects the fluid exchange. As is evidenced in the tissue photographs (*E* to *H*, fig.4) and the fluid intake curves (fig. 6) for *dog* 290C, the stalk can be successfully cut through at its junction with the hypothalamus without precipitating more than a barely perceptible tendency toward a latent diabetes insipidus. Frequently, however, when this procedure is carried out a latent diabetes insipidus is present, and occasionally a mild permanent residual diabetes insipidus occurs even in the absence of demonstrable gross anatomical infringement upon the hypothalamus (21, 22, 24).

The results are also, in the main, variable when total hypophysectomy is made certain at the risk of, or by purposely, infringing upon the hypothalamus. For pur-

poses of description I shall divide these experiments into the categories, total hypophysectomy plus mild, moderate and drastic infringement upon the hypothalamus. The terms mild, moderate and drastic refer to the relative size of the gross tissue defect incurred rather than the functional result.

Total hypophysectomy plus a mild infringement upon the hypothalamus has been accomplished without a residual diabetes resulting. I have not as yet accomplished this without there being a definite latent diabetes insipidus present. Such results are illustrated in the instance of *dog 15 Br* in figures 4 and 6. Following this same procedure, however, there frequently is a mild or moderate residual diabetes insipidus which is preceded by a typical latent period. This is illustrated for *dog 16 Br* in figures 5 and 7. Again following an equivalent tissue defect I have encountered a striking

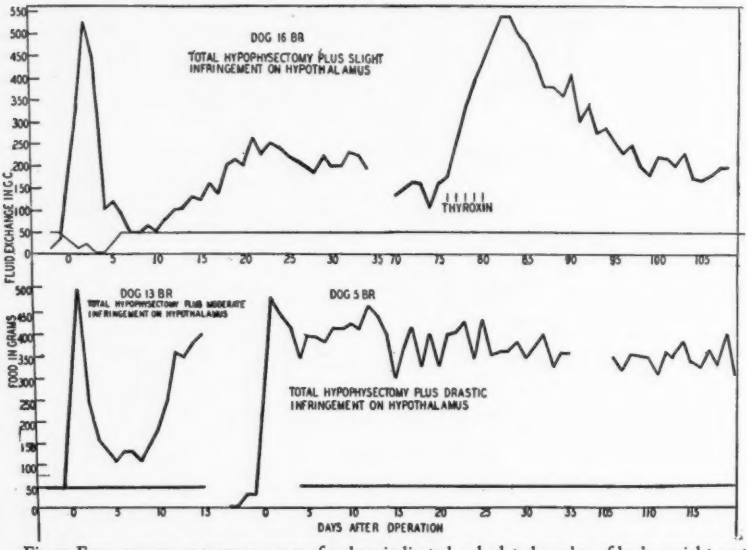

DOG 16 BR
TOTAL HYPOPHYSECTOMY PLUS SLIGHT INFRINGEMENT ON HYPOTHALAMUS
THYROXIN
FLUID EXCHANGE IN C.C.
DOG 13 BR
TOTAL HYPOPHYSECTOMY PLUS MODERATE INFRINGEMENT ON HYPOTHALAMUS
DOG 5 BR
TOTAL HYPOPHYSECTOMY PLUS DRASTIC INFRINGEMENT ON HYPOTHALAMUS
FOOD IN GRAMS
DAYS AFTER OPERATION

Fig. 7. Food and fluid intake curves for dogs indicated, calculated per kg. of body weight on the basis of operative weight. Fluid intake in each instance is graphed by the heavy line and the food intake by the light line. Note the immediate and continually maintained striking polydipsia in *dog 5 Br*, while *dogs 13 Br* and *16 Br* both exhibit early temporary regressions in the fluid exchange to the normal level.

diabetes insipidus which appeared immediately after operation and persisted with no transitory return to or toward the normal. Such an animal is not illustrated but the curve type (fluid exchange) is illustrated in figure 7 for *dog 5 Br*.

Total hypophysectomy plus a moderate infringement upon the hypothalamus has never been accomplished without a permanent diabetes insipidus resulting. However, such a procedure has been successfully carried out wherein a typical latent period has broken the early course of the diabetes insipidus. Such an experiment is illustrated in the instance of *dog 13 Br* in figures 5 and 7.

Total hypophysectomy plus a deliberate drastic infringement involving the anterior hypothalamus has invariably yielded an immediate, persistent polydipsia uncomplicated by a latent period. This type of response is illustrated in figures 5 and 7 for *dog 5 Br*.

DISCUSSION

Antidiuretic-elaborating tissue. The results described above demonstrate that the pars nervosa, in its entirety, is not always essential for the maintenance of a normal

fluid exchange in the cat and dog. Certainly there is little likelihood that the fragmentary peripheral rim of pars nervosa tissue which remained in the cat experiments contained functional secretory elements (see *B* to *G* in fig. 2 and compare with *I* to *N* in fig. 2), whereas the complete isolation of the median eminence was assured by actual removal of a considerable amount of the ventral middle tuberal region of the hypothalamus. In *dogs* 290C and *15 Br* the removal of the pars nervosa tissue was undoubtedly complete, yet they exhibited no residual diabetes insipidus. If the early transitory return of the fluid exchange to, or toward, normal ('latent period') reflects activity of antidiuretic-elaborating tissue then the experiments for *dogs 16 Br* and *13 Br* signify that in the dog also a substantial amount of the ventral middle tuberal tissue can be destroyed in addition to total removal of the tissue of the pars nervosa without eliminating antidiuretic-elaborating activity. In this connection it is pertinent to point out that the onset of permanent diabetes insipidus is always preceded by a latent period when precipitated by anterior hypothalamic lesions, regardless of the extent of the tissue defect, when the hypophysis has not been removed. It therefore seems highly probable that this transitory return of the fluid exchange to, or toward, normal is due to a transitory activity of remaining antidiuretic elaborating tissue and conversely that the absence of a latent period evidences the absence of any such tissue remaining functional, even transiently.

If one assumes first, that diabetes insipidus is an antidiuretic deficiency phenomenon, and second, that a given deficiency in the antidiuretic principle (possibly the antidiuretic-free state) invariably results in an increased water exchange, then it must be acknowledged that tissue other than the pars nervosa of the hypophysis possesses antidiuretic elaborating powers. Therefore, these results tender strong support to the contention first advanced by Abel and later supported by Sato and Trendelenburg, that the tissue of the anterior hypothalamus possesses potent antidiuretic-elaborating powers. It is to be recalled that Abel and Geiling (25) obtained pituitrin by extracting the anterior hypothalamic tissue of normal sheep, while Sato (26) and Trendelenburg (27) obtained pituitrin by extraction of the anterior hypothalamic tissue of normal dogs, but gathered a greater yield from the same tissue of hypophysectomized dogs. On the basis of experiments with *dogs* 290C and *15 Br*, I see no reason to suppose as Sato suspected that the extra-hypophysial antidiuretic-elaborating tissue normally possesses only latent elaborating powers. At the same time, there is no reason to doubt that the anterior hypothalamic elaborating power becomes enhanced in the presence of hypophysectomy as Sato's experiments indicate.

The usual objection to Abel and Geiling's and to Sato's and Trendelenburg's extraction experiments, namely, the probability of diffusion of the active substances from neighboring elaborating hypophysial tissue (remnants in the case of Sato's experiments), is ruled out in the present experiments because all hypophysial tissue including the recently much discussed median eminence was unquestionably completely removed. In fact one might now reverse this argument and ask whether the successful extraction of pituitrin from pars nervosa tissue might not result from diffusion of the active substance from the elaborating hypothalamic focus to the tissue of the pars nervosa. The yields obtained from extraction per unit weight of each of these tissues (26), infundibular process and anterior hypothalamus, argue against such an interpretation and indeed strongly indicate that the pars nervosa is not only intimately associated with active elaboration of this substance, but also plays the more prominent rôle in this respect. Nevertheless, in view of the fact that the pars nervosa can be removed in its entirety without disturbance of an animal's fluid exchange, it is to be borne in mind that the the only direct indication that the antidiuretic substance is actively elaborated by the pars nervosa tissue is that derived from extraction experiments. Thus Osborne and Vincent (28) obtained a greater yield of pituitrin from ex-

tracting the tissue of the central portion of the infundibular process than when they extracted tissue from the peripheral region including pars intermedia tissue. Also Atwell and Marinus (29) obtained pituitrin by extracting uncontaminated infundibulum tissue. Finally, it is well known that Geiling and associates (30, 31, 32) have shown that pituitrin is obtainable from the infundibular process of the bird and whale, which are not complicated by an epithelial investment.

Antidiuretic-elaborating process. Granting that there is an extrahypophysial antidiuretic-elaborating focus, it is of considerable interest to speculate regarding the nature of the elaborating process.

One thinks at first in terms of secretory elements. Scharrer (33), on the basis of cytological studies, postulates the existence of hypothalamic secretory neurons. Finley (34) has called attention to the remarkably rich blood supply of the supraoptic and paraventricular nuclei, which in turn could well support the idea that there are secretory elements in these nuclei. The pituicytes have recently been sponsored specifically as being the antidiuretic secretory elements (16). As far as is known, however, neither nerve cell bodies nor pituicytes are common to both the pars nervosa and the hypothalamus. Very recently Hare et al. (35), on the basis of ingenious experiments, have questioned Gersh's interpretation of his results with respect to the pituicytes.

Gaupp and Scharrer (36) early advanced the supposition "that the supraoptic nuclei not only regulate the activity of the posterior lobe but are themselves capable in the absence of the hypophysis of taking over the antidiuretic function of the posterior lobe" [Fisher's (37) quotation of Gaupp and Scharrer's view]. In view of the fact that the cells of the supraoptic nuclei do not invariably degenerate following section of the infundibular nerve fibers, illustrated in the instance of *cat 116*, such a possibility can not be dismissed as abruptly as does Fisher (37). In this connection I have encountered marked (50 to 80% by gross approximation) disappearance of the cells in the supraoptic nuclei in the absence of a residual diabetes insipidus in both the cat and dog, while in other instances, in the presence of a well marked diabetes insipidus, a considerable number of healthy looking cells have remained. From such observations I have gained the general impression that the degeneration of the cells in the supraoptic nuclei is not primarily the causative factor in precipitating diabetes insipidus.

On the other hand, the pars nervosa and supraoptic crest do possess in common a "similarity in regard to the selective staining by acid dyes as well as vascularity" as has been demonstrated by Wislocki and King (1). The possibility therefore arises that these two regions may possess antidiuretic-elaborating powers in common. If this is the case, the ease in precipitating diabetes insipidus as a result of manipulation in the region of the proximal stalk would be explainable on the basis of the vulnerability of the vascular supply to the supraoptic crest to such manipulation. If the elaboration of the antidiuretic principle proves to be associated with the distinctive vascularity of the pars nervosa and supraoptic crest, it seems doubtful that the elaborating process in its final stage is a true secretory process, as we ordinarily think of active secretion (38).

Denervation theory. The proponents of the denervation theory have universally held that normal water metabolism is dependent upon tonic impulses passing to the hypophysial tissue from nerve cells located in the hypothalamus. The theory as thus delimited implies the existence of an endocrine elaborating process as dependent upon neurogenic activity as is striated muscle and more dependent on such activity than is smooth muscle of arterioles. Indeed the theory, in its most recent form, would have the very existence of the effector organ as dependent upon the cell bodies of the innervating neuron as the axon itself! It is quite true that should such striking deviations

from the usual exist it would not be surprising to encounter them associated with such a basically tonic function as control of water metabolism.

At the expense of stressing the obvious, a few remarks regarding the evaluation of the data bearing on the testing of the denervation theory are in order because of the present wide acceptance of this concept as an established fact even in the face of evidence to the contrary. In evaluating the evidence for the support of any theory, it is of utmost importance to have clearly in mind the question being put to test in order to recognize, in sharp relief, whether the results give or do not give a decisive answer. In this case the fundamental question being put to test is not whether permanent diabetes insipidus can be precipitated by anterior hypothalamic lesions, a fact that has been demonstrated again and again in irrefutable form, but whether a permanent diabetes insipidus precipitated by such lesions is the result of the elimination of tonic nerve impulses passing from the hypothalamus to the hypophysis by way of certain or all of the infundibular nerve fibers. The fact that the precipitating lesion is so situated that a prominent component of the infundibular bundle of fibers is usually or always severed, gives, in the absence of data to the contrary, presumptive support to the denervation theory, but surely does not give decisive proof.

In this connection attention is called to the fact that in the experiments of the Richter and Ranson groups, upon which credence for the denervation theory is largely built, it is not at all certain that diabetes insipidus was always precipitated following the severance of the anterior component of the infundibular group of fibers. In those experiments total hypophysial denervation was never attained, nor even attempted, and for the instances in which diabetes insipidus failed to follow the attempted placing of appropriate lesions, it was more or less routinely assumed that sufficient of the antidiuretic fibers remained unsevered. In preparations in which the lesions are confined to the anterior hypothalamus, all of the fibers coursing in the posterior infundibulum and many sweeping down laterally to enter the distal ventral infundibulum remain intact, and there is no anatomical assurance that the entire anterior hypothalamic component is or is not severed.

On the other hand, the successful severance of all the nerve fibers which enter and traverse the infundibulum (which of necessity must include the hypothetical antidiuretic fibers), without precipitating diabetes insipidus, casts grave doubt upon the correctness of the denervation hypothesis as it is defined and qualified above and in our previous papers.

It is pertinent to point out, however, that if extrahypophysial tissue possessing antidiuretic elaborating powers exists, then there is, as yet, no evidence which necessarily supports, or for that matter argues against, the denervation theory as a causative factor in diabetes insipidus, because anterior hypothalamic secretory elements might be just as dependent upon tonic neurogenic influences as such elements located in the pars nervosa.

Consideration of factors that might possibly complicate denervation or removal experiments. We must, however, carefully scrutinize experiments of the foregoing nature for factors that might have clouded our results.

First, there is the matter of the possibility of incomplete severance of the infundibular fibers. As has been previously pointed out, we have taken meticulous care in making certain that our denervations were absolutely complete. From the outset our criteria for complete denervation have included not only the demonstrated absence of nerve fibers in the pars nervosa as evidenced by controlled silver staining, but also the complete demarcation of the hypophysial stalk from the hypothalamus by the scar tissue of the lesion as shown by silver, Nissl, and Pal-Weigert staining of appropriate serial sections. Our operative procedures have always been designed so that there would never be any question of fragments of the proximal infundibulum (median

eminence) being left proximal to the lesion scar. As pointed out above, histological studies have verified our successful execution of these particular procedures. Surely the photographs of sections from *cat 116* in figure 2 should convince the most doubtful regarding total severance of the infundibular nerve fibers as well as the complete isolation of the median eminence. The photographs of sections from dogs *290C* and *15 Br* in figure 4 should be equally convincing with regard to there being no remnant of the median eminence left intact.

Secondly, there is the matter of possible complication due to the absence of the pars anterior. Thus the failure of a residual diabetes insipidus to follow in the wake of total hypophysectomy [specific experiments being those of Reichert and Dandy (39) in the dog and Ingram and Fisher (13) in the cat] has been explained on the basis of von Hann's (40) concept that the appearance of diabetes insipidus is dependent upon an intact functional pars anterior. During the course of our first series of experiments, we thought seriously that the hypothalamic manipulation might in some manner impair the function of the pars anterior sufficiently so as to obscure any antidiuretic deficiency. Since that time, both White and Heinbecker (22) and I (21, 24) have shown by attacking this specific question directly, that the von Hann hypothesis does not hold to the extent that total impairment of the pars anterior would complicate the interpretation of our results. The fallacy of the von Hann concept, accepted as rigidly as Ingram and Fisher do in their discussion, is demonstrated particularly well in the instances of *dogs 290C* and *5 Br*. *Dog 290C* had an uncomplicated total hypophysectomy and exhibited no residual or definite latent diabetes insipidus while *dog 5 Br* had a total hypophysectomy complicated by drastic infringement upon the anterior hypothalamus exhibiting a striking persistent diabetes insipidus. Yet they suffered equally in approximately a 30 per cent lowering of basal metabolism as a result of their respective operative procedures[4].

The fact that hypophysectomy is compatible with diabetes insipidus does not detract, per se from the use of certain metabolic-raising substances [thyroid (41, 42) anterior lobe extract (43, 44) and thyroxin (45, 46)] as tools in detecting latent diabetes insipidus. We have found, however, that these substances must be used in this connection with some reservation. First, in our hands, they have been decidedly more dependable in the dog than in the cat. Secondly, overdosage will eliminate an already established diabetes or prevent the usual response in latent diabetes insipidus. The range between an effective stimulating dose and an outright overdosage is not great. Nevertheless, when due care is taken in testing for latent diabetes insipidus, the failure of a striking rise in the fluid exchange can be accepted with confidence as the indication of the absence of a latent diabetes insipidus.

Thirdly, another avenue whereby diabetes insipidus evaluation experiments may be complicated is through the health factor. Fisher and Ingram (42) have pointed out that diabetes insipidus in cats may disappear with the onset of a disease such as distemper. We have seen in several instances a moderate diabetes insipidus disappear with the onset of a siege of distemper or mange, when the infections were sufficiently severe to put the animals off their food, and to return with the animal's recovery. We have assumed that spontaneous normal appetite with maintenance of body weight is an adequate criterion of the good health of the animal. However, in order to be certain that some unknown health factor was not entering into our experiments we

[4] I am indebted to Doctor John M. Bruhn for the metabolism studies on these dogs. The method used in determining basal metabolism has been described by him (46). This paper also gives further specific metabolism information concerning *dogs 5 Br* and *290C*, which are, respectively, *dogs 2* and *5* in his paper. "Since the calories per kilogram of body weight of normal dogs falls sharply with increasing weight, the decrease was calculated as the percentage lowering of metabolism from the predicted value of normal dogs of similar weight rather than from the preoperative level. Had this method of calculation not been used the decrease in metabolism would be even more pronounced, because increases in weight occurred in both animals after operation."

made the experiment described for *cat* 94C. This cat before operation was conditioned to living free in the environs of the medical building and the neighboring fraternity houses. Following operation it was allowed to return to these environs and after an appropriate period of time, when in excellent condition, its return to cage regime revealed no evidence of any deviation from the normal in its fluid exchange, as illustrated in figure 1.

Lastly, there is a possible complication that appears when one considers the great variation in the severity of the diabetes insipidus encountered following what appears to be homologous hypothalamic lesions. We have pointed out that it would be logical to expect that animals of the same species having the same deficiency in the antidiuretic principle would exhibit comparable increases in their fluid exchange per unit of body weight. During the past years, we have carried out several groups of experiments designed to determine whether this assumption was or was not correct. The data so far have not given a decisive answer. Therefore, the question is raised as to whether an antidiuretic-free state is always incompatible with a normal fluid exchange. If one assumes that the elaboration of the antidiuretic principle is rigidly confined to the pars nervosa then the data presented in this paper would force the conclusion that the antidiuretic-free state is not invariably incompatible with a normal fluid exchange. If this is true, one should be able to perform total hypophysectomy plus drastic infringement upon the anterior hypothalamus without eliciting diabetes insipidus with the same incidence as in either total hypophysectomy or the selective elimination of or total denervation of the pars nervosa depending upon the particular approach being utilized. This decidedly has not been our experience nor that of White and Heinbecker (47). Referring to White and Heinbecker's results, it is to be pointed out that their results differ from ours only in that total removal or total isolation of the pars nervosa invariably resulted in diabetes insipidus in their hands, whereas we have successfully executed these procedures without eliciting diabetes insipidus. We have, however, never failed to encounter a persistent diabetes insipidus following total hypophysectomy plus drastic infringement upon the anterior hypothalamus. Thus we are in agreement in believing that a near antidiuretic-free state is incompatible with a normal fluid exchange.

CONCLUSIONS AND SUMMARY

1. The entire hypophysis was isolated from the hypothalamus in the cat, by the removal of the ventral tuberal portion of the hypothalamus with the subsequent disappearance of all but a narrow peripheral rim of pars nervosa tissue, without precipitating a residual or latent diabetes insipidus.

2. Uncomplicated total hypophysectomy was successfully executed in the dog without precipitating a residual or latent diabetes insipidus.

3. Total hypophysectomy plus a mild infringement upon the ventral tuberal hypothalamus was performed without precipitating a residual diabetes insipidus although a latent diabetes insipidus was present.

4. Total hypophysectomy plus a moderate infringement upon the ventral tuberal hypothalamus was accomplished without eliminating the return to normal of the water exchange ('latent period') which precedes the onset of permanent diabetes insipidus.

5. An immediate and sustained diabetes insipidus, unbroken by the appearance of a 'latent period,' invariably followed total hypophysectomy plus drastic gross infringement upon the anterior hypothalamus.

6. It is pointed out that in spite of our success in isolating the entire hypophysis, as well as accomplishing total hypophysectomy, without precipitating diabetes insipidus, we have frequently encountered diabetes insipidus following these same procedures. Indeed, great care must be taken to avoid precipitating diabetes insipidus by

manipulation in the junctional region between the hypophysis and hypothalamus.

7. It is demonstrated that the reduction in metabolism which occurs as a result of hypophysectomy is compatible with a well pronounced diabetes insipidus.

8. The foregoing observations are interpreted as demonstrating, with certain reservations discussed in the body of the paper, the existence of an extra-hypophysial antidiuretic-elaborating mechanism. Further it is strongly indicated that the location of such a mechanism is in the immediate environs of the anterior hypothalamus.

9. Attention is called to the fact that if one accepts the existence of extra-hypophysial elaborating tissue, there is no direct evidence which would necessarily argue for or against the denervation theory as a causative factor in the production of diabetes insipidus.

10. It is demonstrated that retrograde degeneration of the cells of the supraoptic nuclei does not invariably follow section of the nerve fibers passing from the hypothalamus into the infundibulum.

REFERENCES

1. Wislocki, G. B., and L. S. King: *Am. J. Anat.* 59: 421. 1936.
2. Rioch, D. M., G. B. Wislocki and J. L. O'Leary: *A. Research Nerv. Ment. Dis.* 20: 3. 1940.
3. Bucy, P. C.: In Cytology and Cellular Pathology of the Nervous System. W. Penfield, editor. Vol. 2, p. 705. Paul B. Hoeber, Inc., New York, 1932.
4. Keller, A. D.: *Am. J. Physiol.* 133: 346. 1941.
5. Futcher, M. B.: *Am. J. M. Sci.* 178: 837. 1939.
6. Richter, C. P.: *Brain* 53: 76. 1930.
7. Broers, H.: Experimentelle Diabetes Insipidus. Kemink and Son, Utrecht, 1932.
8. Fisher, C., W. R. Ingram and S. W. Ranson: *Arch. Neurol. & Psychiat.* 34: 124. 1935.
9. Ingram, W. R., C. Fisher and S. W. Ranson: *Arch. Int. Med.* 57: 1067. 1936.
10. Cushing, H.: Papers Relating to the Pituitary Body, Hypothalamus and Parasympathetic Nervous System. Charles C Thomas, Springfield, Ill., 1932.
11. Richter, C. P.: *Am. J. Physiol.* 110: 439. 1934.
12. Ingram, W. R., and C. Fisher: *Anat. Rec.* 66: 271. 1936.
13. Ranson, S. W., C. Fisher and W. R. Ingram: *A. Research. Nerv. Ment. Dis.* 17: 410. 1936.
14. Fisher, C., W. R. Ingram and S. W. Ranson: Diabetes Insipidus and the Neurohormonal Control of Water Balance. Edwards Brothers, Inc., Ann Arbor, 1938.
15. Heinbecker, P. and H. L. White: Ann. Surg. 110: 1038. 1939.
16. Gersh, I.: *Am. J. Anat.* 64: 407. 1939.
17. Keller, A. D., and W. J. Hamilton: *Am. J. Physiol.* 119: 348. 1937.
18. Keller, A. D., and W. J. Hamilton: *Arch. Surg.* 37: 760. 1938.
19. Keller, A. D.: *Proc. Soc. Exper. Biol. & Med.* 43: 61. 1940.
20. Bruhn, J. M., and A. D. Keller: *Am. J. Physiol.* 133: 229. 1941.
21. Keller, A. D.: *Am. J. Physiol.* 123: 116. 1938.
22. White, H. L., and P. Heinbecker: *Am. J. Physiol.* 123: 213. 1938.
23. Cushing, H.: *Boston M. & S. J.* 168: 901. 1913.
24. Keller, A. D.: *Proc. Soc. Exper. Biol. & Med.* 39: 555. 1938.
25. Abel, J. J.: *Harvey Lect.* 19: 200. 1923.
26. Sato, G.: *Arch. f. exper. Path. u. Pharmakol.* 131: 45. 1928.
27. Trendelenburg, P.: *Klin. Wchnschr.* 7: 1679. 1928.
28. Osborne, W. A., and S. Vincent: *Brit. M. J.* 1: 502. 1900.
29. Atwell, W. J., and C. J. Marinus: *Am. J. Physiol.* 47: 76. 1918.
30. DeDawder, A. M., L. Tarr and E. M. K. Geiling: *J. Pharmacol. & Exper. Therap.* 51: 142. 1934.
31. Geiling, E. M. K.: *Bull. Johns Hopkins Hosp.* 57: 123. 1935.
32. Geiling, E. M. K., and M. R. Lewis: *Am. J. Physiol.* 113: 534. 1935.
33. Scharrer, E.: *Ztschr. f. d. ges. Neurol. u. Psychiat.* 145: 452. 1933.
34. Finley, K. H.: *A. Research Nerv. Ment. Dis.* 18: 94. 1938.
35. Hare, W. K., R. C. Hickey and R. S. Hare: *Am. J. Physiol.* 133: 316. 1941.
36. Gaupp, R., Jr., and E. Scharrer: *Ztschr. f. d. ges. Neurol. u. Psychiat.* 153: 327. 1935.
37. Fisher, C.: *Endocrinology* 21: 19. 1937.
38. Gersh, I.: *A. Research Nerv. Ment. Dis.* 17: 433. 1938.
39. Reichert, F. L., and W. E. Dandy: *Bull. Johns Hopkins Hosp.* 58: 418. 1936.
40. von Hann, F.: *Frankfurt Ztschr. f. Path.* 21: 337. 1918.
41. Mahoney, W., and D. Sheehan: *Am. J. Physiol.* 112: 250. 1933.
42. Fisher, C., and W. R. Ingram: *Arch. Int. Med.* 58: 117. 1936.
43. Keller, A. D.: *Proc. Soc. Exper. Biol. & Med.* 36: 787. 1937.
44. Keller, A. D.: *Proc. Soc. Exper. Biol. & Med.* 38: 13. 1938.
45. Bruhn, J. M.: *Am. J. Physiol.* 126: 448. 1939.
46. Bruhn, J. M.: *Am. J. Physiol.* In press.
47. Heinbecker, P., and H. L. White: *Am. J. Physiol.* 133: 582. 1941.

STUDIES ON THE CHEMISTRY OF THE MELANOPHORE HORMONE OF THE PITUITARY GLAND[1]

I. CRITICAL EVALUATION OF ASSAY METHODS

N. O. CALLOWAY, ROBERT M. McCORMACK AND NERISSA P. SINGH

From the Department of Pharmacology of the University of Chicago
CHICAGO, ILLINOIS

AN ESSENTIAL PREREQUISITE for the concentration and isolation of a hormone is a simple and accurate method of assay. The purpose of this paper is to evaluate some of the variables in the available (1) assay methods and to report a simple controlled procedure for the quantitative determination of the melanophore hormone of the pituitary gland. We further wished to ascertain whether or not the alkali-potentiated hormone could be validly compared with standard pituitary powder.

EXPERIMENTAL PROCEDURE

After having tried various test animals, such as the chameleon (*Anolis*), the salamanders (*Amblystoma Mexicanum* and *A. tigranum*) and the red bellied dace (*Chrosomus eos*), we decided to employ exclusively the hypophysectomized frog (*Rana pipiens*) because of its ready availability, hardiness and ease of hypophysectomy (1, 2, 3).

The frogs were obtained from commercial supply houses and kept in a deep sink with slowly running water. No food was given to the frogs at any time. The animals were hypophysectomized a few days after arrival. They survived after operation for a period of a few weeks in summer and several months in winter.

The hypophysectomies were carried out according to the method described earlier (4), except that we did not use the antiseptic nor replace the plate of bone in the base of the skull. Neither of these steps was found necessary.

In successfully operated animals, the melanophores contract in about two hours. Maximum contraction has occurred well within 24 hours. Irregular assay results were obtained when the animals were used 3 to 6 hours after operation. However, there is no refractory period, and they give a response to hormone injection within an hour after operation. Two days is ample time to allow for postoperative recovery; the weaker ones usually die within this time.

The frogs could be used repeatedly if 24 hours elapsed between complete contraction and re-injection. In the dosage ranges used no quantitative difference could be observed in response of frogs recently hypophysectomized, those previously operated and those repeatedly used. The only exception noted was in the cases where very large doses had been given previously.

On the second day after operation the frogs were closely examined. All animals displaying flatulence, edema, hemorrhage or lack of vigor were eliminated. The se-

Received for publication December 29, 1941.
[1] This investigation was supported by grant from the Committee on Research in Endocrinology, National Research Council.

lected frogs were then examined microscopically. Only those frogs were kept for quantitative use in which the melanophores were *fully contracted to circular or oval shapes*.. Operated animals in which the melanophores[2] had even minute stellate processes were rejected. Such rejected animals could be used for qualitative determinations only.

After hypophysectomy the frogs were kept in the laboratory in small individual wire cages set in pans of water. The water was changed daily and the pans washed. The water was kept deep enough so that the frog could comfortably keep his nostrils above the water. This is important, especially in hot weather, since shallow water vitiates the results. The pans were kept close together so that the temperature of the animals was approximately the same.

The only disease with which we had difficulty was 'red leg.' This can be adequately controlled by generally good sanitation, and using hot water to clean infected cages. The use of copper sulphate, one part in 40,000 parts of water, in the pans will check red leg epidemics if the frogs are exposed to the copper sulphate for 24 hours.

All glassware was washed in water, cleaned with sulfuric-chromic acid mixture, washed several times with hot water and kept out of the dust in a drying oven. Occasionally pieces of apparatus were washed with a small amount of water which was injected into a frog to test for pituitary powder dust which sometimes contaminates the apparatus or an entire laboratory where large quantities of pituitary powder are handled. This procedure is essential in laboratories in which the powders are prepared from acetone-desiccated glands.

Extractions of all materials were made by weighing on a torsion balance to the nearest 0.02 mg. The material was quantitatively transferred to a small mortar and triturated with the solvent, boiled for one minute, filtered and diluted. Transfers were made with Pasteur pipettes with rubber bulbs. Volume measurements were made in graduated centrifuge tubes.

The accurate control of hydrogen ion concentration was found to be of the utmost importance. Hydrogen ion concentration was determined by a glass electrode with necessary corrections. The lower pH value was obtained by using a 0.25 per cent solution of acetic acid in distilled water. The solution of higher pH was a 0.04 molar solution of trisodium phosphate.

Injections were made into the ventral lymph sac of the frog through the mouth. A one-inch, 24-gauge needle was found satisfactory.

It was found that the melanophores of the webbing of the foot were variable in their response. Size or distance separating the melanophores and the frog's general condition were factors. Therefore, late in the experiments, we adopted the policy of reading the melanophores on the medial side of the frog's leg just below the insertion of the gastrocnemius muscle and above the smallest toe. There are small patches of melanophores in this region that are constant in position, sensitive to circulation, easily observed and positively located at each reading, since definite anatomical landmarks are always present.

Reading was done under a binocular dissecting microscope using 48 mm. objectives and 10 × oculars. Such a combination gave sufficient detail and also a satisfactorily sized field.

In observing the responses, a variety of methods were tried. Since all melanophores expand at a uniform rate regardless of dose, the rate of expansion of the melano-

[2] The term 'melanophore' is used to indicate the black portion of the melanocyte as has been suggested and discussed fully elsewhere (1).

phores could not be used as a criterion of hormone activity. The duration of expansion at any stage was untenable since stages of expansion are arbitrary and illdefined. The rate of contraction could only be measured by a micrometric method. Gross color changes vary from one frog to another, and greenish and brownish skin colors interfere with gross observations in quantitative work.

Therefore, we adopted as the criterion of activity the length of time which is required for the melanophores to return to full contraction after injection. This we have termed contraction time. The readings were made every 15 minutes during the first hour and during any critical period of contraction, such as at the end of an assay. However, when the assay lasts over 200 minutes, 30-minute intervals are satisfactory since the error introduced by these intervals is not significantly greater than the allowable error of the assays.

All experimental data were checked at least once on different days with freshly prepared solutions. In these checks it was not possible to get exactly identical data, but the relative values obtained between the standard and the compared solutions were the same.

DISCUSSION OF RESULTS

In order to ascertain the optimum conditions for the assay, we used a large range of doses and observed a series of animals closely throughout the period of the response. It was found that the dose should always be sufficient to give a response which lasted for a minimum of 60 minutes. If the response is of quite short duration, unless the animals are read frequently, the error caused by the interval between readings becomes significant. Further, if the response is less than an hour's duration, the velocity of expansion and contraction becomes significant, and it is not possible to discern the minimum duration of the effect. Similarly a maximum duration of expansion had to be fixed since beyond a certain duration of response, the errors became larger and the variations became greater. Thus, the exponential relationship of the plotted data was distorted. Therefore, it was experimentally determined that a contraction time of approximately 240 minutes should not be exceeded in quantiative comparisons.

Furthermore, there is some type of physiological lag in contraction when larger doses are given so that the response curve is not only distorted but with large doses the frog does not regain his light color of contracted state of his melanophores even after two weeks. Since it was desirable to construct curves for interpretative purposes from the data, we utilized doses which gave curves of an exponential nature.

The most useful graphs were obtained by plotting the logarithm of concentration of hormone expressed as micrograms of standard pituitary powder against time of return to full contraction expressed in minutes. This gave a straight line when the dosages were such that the contraction time was between 60 and 240 minutes, when the volume of the dose was constant at 0.2 cc. of solution. The standard solutions were made up 1 mg. of standard powder per cc. of solvent and the necessary dilutions made therefrom.

The influence of concentration of the hormone is readily seen from figure 1. It will be noticed that for the material extracted at approximately a pH of 3, the slope of the line is greater than at the higher pH of 12. This indicates that the length of response is less for a given dose of hormone when the material was extracted at a pH of 3. When the extraction was made at a pH of 12 the slope of the line was such that at low concentrations the response of the acid-extracted and base-extracted hormones could be identical while the higher the concentration the farther apart were the responses. This may account for the discrepancies re-

ported in the literature between the work of various investigators who have sought to determine the degree of potentiation in alkali treated material.

This indicates that identical hydrogen ion concentrations must be maintained in all assays for both the reference standard and the unknown material. The assay may be carried out at any desired pH. Care must be exercised to maintain the pH of all solutions at a value no higher than is to be used for the basis of the assay. For example, any increase in pH introduces the factor of potentiation which vitiates the results even though the pH is subsequently lowered.

The surprising observation was made that the total dose of hormone was not the only factor involved in the duration of response. Thus, figure 2 shows that when the

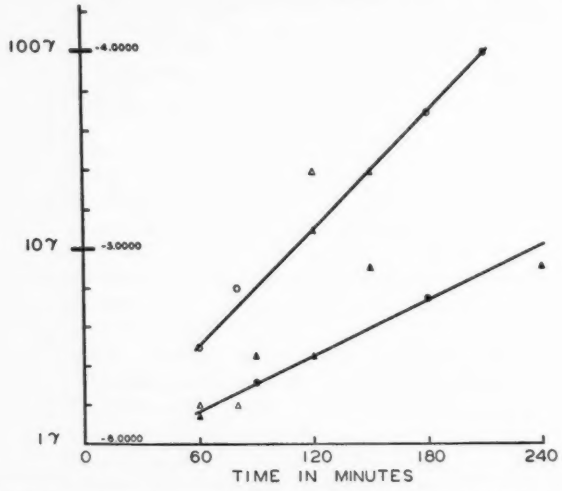


Fig. 1. Effect of increasing concentration of hormone on contraction time, at pH 3 and pH 12 Dose: 0.20 cc. of beef pituitary powder extract, concentrations as indicated. Ordinate: left, concentration in μg. per cc.; right, logarithm of concentration in grams per cc. Abscissa: Contraction time of the melanophores expressed in minutes. ○ = 2 frogs at pH 3; △ = 1 frog at pH 3; ● = 2 frogs at pH 12; ▲ = 1 frog at pH 12.

dose of hormone is increased 30 times by increasing the volume and not the concentration, the response only increases one-third to one-half for the lower pH and one to one and one-half times for the higher pH. This strikingly indicates that some physiological factor, such as absorption, elimination or inactivation, has become so pronounced as to render increasing volume of dose useless as an assay method. Thus, the volume of injected solution must be kept constant and the concentration varied. Incidentally, many investigations have failed to take cognizance of this important fact.

All of these facts illustrate that the nature of the response is different at the two pH values and indicate a possible difference physiologically in the potentiated and nonpotentiated hormone (5) as has been suggested previously (6). It is assumed, throughout, that extraction at pH 3 yields the nonpotentiated form of the hormone.

To ascertain the influence of temperature, a series of studies were made in which two groups of frogs were treated identically, except that one group was maintained at 23°C. and the other at 33°C. during the assay. The melanophores of the animals at the higher temperature contracted in half the time required by those at the lower

temperature. Therefore, it is necessary to maintain the temperature fairly constant on all animals used during an assay.

The variations in light intensity in the ordinary laboratory have no effect on the assay.

When 4 frogs were used to locate each point, essentially the same accuracy was obtained as when 2 frogs were used. In fact, when assays were made on four different days using a total of 8 frogs for a single point the greatest deviation observed was ±10 per cent from the mean. Therefore in view of these two facts we used 2 frogs to locate each point.

However, in order to evaluate response accurately, it was found necessary always

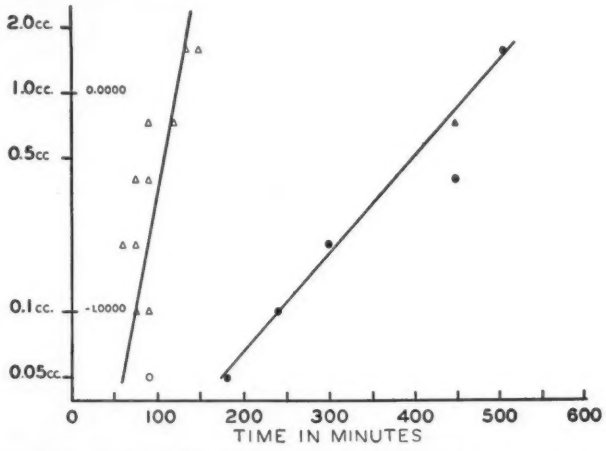


Fig. 2. Effect of increasing volume of hormone extract on contraction time, at pH 3 and pH 12. dose: 5 μg. per cc. beef pituitary powder extract, volume as indicated. Ordinate: left, volume in cc.; right, logarithm of the volume. Abscissa: contraction time of the melanophores expressed in minutes. ○ = 2 frogs at pH 3; △ = 1 frog at pH 3; ● = 2 frogs at pH 12; ▲ = 1 frog at pH 12.

to use at least three different concentrations and to plot the data for both the reference standard and the unknown so that the slope of the lines could be observed. The data are not comparable if the angles of inclination of the slopes vary more than the desired accuracy of assay. Thus, a minimum of six animals should be used for each unknown and six for the reference material. This gives three points to establish each graph.

In order to determine the activity of a given material after having the experimental data, it is necessary to read either the concentrations at a fixed time of contraction or to read the time of contraction at any given concentration. The two factors are then related to each other by simple logarithmic algebra so that for each assay the logarithmic relationship between time of contraction and concentration of hormone must be determined.

For example, in figure 3 are plotted data from two determinations on the same material run simultaneously. The error present represents the total error using 6 frogs in each series at a pH of 3. Two frogs were used for each concentration. The data were plotted and the lines drawn by inspection.

In order to determine the relative activities of the two materials any arbitrary time may be selected such as 120 minutes. The concentrations which gave identical

responses cannot be read directly since the ordinate is graphed as the logarithm of the concentration. Thus it is necessary to read these logarithms from the graph. Then each antilogarithm is found. The difference of these antilogs, which is the difference in the concentrations necessary to give a 120-minute response, is the error of the determination. For this particular determination no special precautions were used and to illustrate its validity a person who had never made an assay took the readings. The mean error is ± 17 per cent. This error may be further reduced by practice in reading, increasing the number of animals, and by employing only calibrated apparatus, and giving strictest attention to quantitative technique.

It should be pointed out that this is, as far as we are aware, the only method which

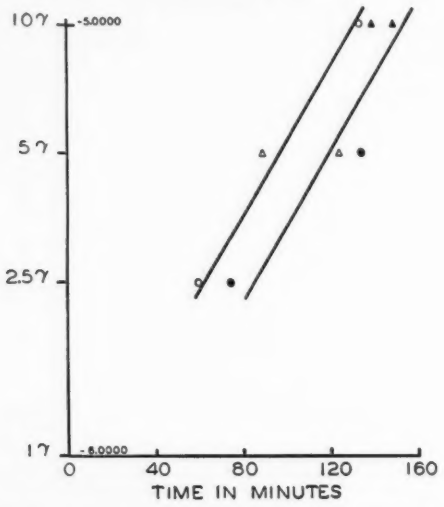


Fig. 3. Evaluation of assay method for the melanophore hormone. Dose: 0.20 cc. of beef pituitary powder extract, concentrations as indicated. Ordinate: left, concentration in μg. per cc. right, logarithm of the concentration in gm. per cc. Abscissa: Contraction time of the melanophores expressed in minutes. ○ = 2 frogs from *Series I*; △ = 1 frog from *Series I*; ● = 2 frogs from *Series II*; ▲ = 1 frog from *Series II*.

has been described in which the hormone can be determined without the tedious and impractical trial and error method of dilutions until the same response is obtained.

A simpler, less exact assay may be carried out by making up the reference material and unknown in exactly the same manner and at the same pH, then injecting two frogs with each solution. The two contraction times may then be approximately related, to give the actual dosage by use of a logarithmic factor previously graphically determined in a similar assay under related but more detailed conditions. Such an assay assumes that the usual variables are constant and that previous data hold for the present determination. While such assumptions are dangerous, this type of assay is often sufficient for roughly quantitative determinations. Under no circumstances is it possible to compare response and dosage directly since these factors are always related logarithmically!

Throughout our work we have adopted the melanophore-hormone unit as the activity of one microgram of U.S.P. XI posterior pituitary powder as defined earlier

(2). However, we suggest that the unit be not confined to alkali potentiated material as was suggested (2), but rather the unit be fixed as one microgram of posterior pituitary powder and that any given assay be made at a determined hydrogen ion concentration.

SUMMARY

It is possible to quantitatively determine the potency of melanophore hormone extracts using hypophysectomized *Rana pipiens* as the test animal.

It was found essential to control the hydrogen ion concentration at all times in an assay and further it was found necessary to always compare the known and unknown at the same pH.

It was found that the most satisfactory assay was obtained using increasing concentration as a variable and not increasing volume of hormonal extract.

It is necessary to control variations of temperature.

The assay as described eliminates the impractical procedure of attempting to dilute a known and an unknown until they yield identical responses.

We express our appreciation to Dr. E. M. K. Geiling for his critical supervision. We also wish to thank Dr. Morris Rosenfeld, visiting associate professor of pharmacology, for helpful suggestions and criticisms.

REFERENCES

1. Oldham, F. K., N. O. Calloway and E. M. K. Geiling: *Ergebn. d. Physiol.* In press. 1942.
2. Teague, R. S.: *Endocrinology* 25: 962. 1939.
3. Teague, R. S., R. O. Noojin and E. M. K. Geiling: *J. Pharmacol. & Exper. Therap.* 65: 115. 1939.
4. Hogben, L. T.: *Quart. J. Exper. Physiol.* 13: 177. 1922.
5. Calloway, N. O., R. M. McCormack and E. M. K. Geiling: *Endocrinology* 30: 430. 1942.
6. Stehle, R. L.: *J. Pharmacol. & Exper. Therap.* 57: 1. 1936.

STUDIES ON THE CHEMISTRY OF THE MELANOPHORE HORMONE OF THE PITUITARY GLAND

II. THE EFFECT OF HYDROGEN ION CONCENTRATION ON POTENTIATION[1]

N. O. CALLOWAY, ROBERT M. McCORMACK AND E. M. K. GEILING

From the Department of Pharmacology, University of Chicago
CHICAGO, ILLINOIS

MOST OF THE PRINCIPLES of the posterior lobe of the pituitary gland are destroyed at lowered hydrogen ion concentrations (1). However, the melanophore hormone of the pituitary gland is not only stable at the higher pH ranges but is actually enhanced in its activity (2). This unusual property has been studied from various angles by many investigators (3).

While studying the general behavior of the hormone, we found it necessary to observe the effects of various hydrogen ion concentrations. These observations are reported in this paper.

We have also utilized this information in interpreting the possible nature of the potentiation phenomenon, which is a most unusual and intriguing property of this hormone.

EXPERIMENTAL METHODS

The methods used were in general the same as those described in the preceding paper (4). The pH was measured by electrometric methods using a glass electrode and correcting for the variations at higher pH values with a hydrogen electrode. The solutions were all buffered and made from 0.04 molar citric acid and 0.04 molar trisodium phosphate for the higher values and disodium phosphate for the lower ones. In general, a color indicator (methyl red) was used to obtain neutrality before injection. However, it was later found that since the original solutions were diluted one part to 100 it was quite unnecessary to neutralize the material at all.

The test material was standard beef posterior pituitary powder prepared according to the U. S. Pharmacopoeia XI. It was always made up so that the final filtered material contained the equivalent of one mg. of powder per cc. When the material was to be neutralized to check the action of the alkaline solutions, the proper allowances were made by weighing the indicated quantity of powder and diluting with distilled water after neutralization.

The only variation from the previously described (4) extraction procedures was that the material was routinely boiled for two minutes before filtering.

RESULTS AND OBSERVATIONS

It was found that when the original 1 mg.-per-cc. extract was diluted one to one

Received for publication December 29, 1941.
[1] This investigation was supported by a grant from the Committee on Research in Endocrinology, National Research Council.

hundred parts of water and 0.25 cc. of the material was injected, a definite and satisfactory response was obtained. The criterion of response was the duration of expansion of frogs' footweb melanophores.

These data were then plotted so that the ordinate was pH and the abscissa was time in minutes.

It will be seen from figure 1 that the increased activity of the hormone at higher pH values is gradual below a value of 7. Above this value the potentiation becomes rapidly greater so that at a pH of 12 it is several times the value at the lower pH values. Any attempt to interpret the number of times greater the response at higher pH values meets with difficulty since there is definitely a different type of response as has been shown (4).

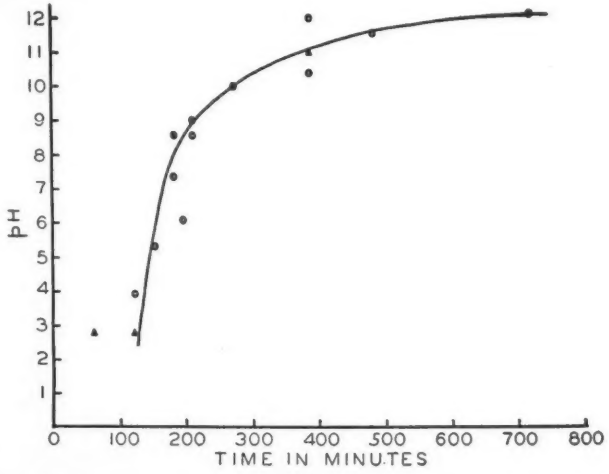


Fig. 1. The potentiation of the melanophore hormone with increasing hydroxyl ion concentration.

Dose: 0.25 cc. of beef pituitary powder, concentration 10γ per cc. Ordinate: pH Abscissa: contraction time of the melanophores in minutes. o = 2 frogs, Δ = 1 frog.

It is possible to determine, for each dose, the relationship between pH and response. But for every dose a curve of slightly different curvature is obtained so that for very dilute solutions we found a small curvature, especially at the lower pH values.

However, at the lower pH values there is definitely a potentiation, which establishes the fact that the potentiation is a function of hydroxyl ion concentration and not a phenomenon which suddenly appears at an extremely high pH. However, we have assumed throughout our work that no potentiation occurs at a pH of 3.

In material stored for one week after the customary preparation, the hormone was found to deteriorate at the lower pH values, unless this was inhibited by a protecting layer of toluene over the material. This indicates that bacterial or mycotic activity may account for the loss of activity at the lower values.

We found that the anterior pituitary lobes both of the whale and of beef showed this same potentiation.

The irreversibility of the process was verified (5) by extracting the material at the pH of 3, shifting the pH to 12 at the boiling point for two minutes, and then

neutralizing. This material was as active as the material extracted at a pH of 12. This also shows that it is not a solubility phenomenon. The material extracted at a pH of 12 was in no way observably altered by changing the pH to 3, heating for 2 minutes and filtering. These same procedures applied to intermediate pH values showed the activity of the highest pH attained in the procedure.

To ascertain whether or not some inhibiting substance was present, a portion of hormone solution was divided into two parts. One was made alkaline (pH 12) for 2 minutes at the boiling point; the other heated similarly at a pH of 3. The two were used separately after neutralizing, and portions of these were mixed so that there were 3 volumes of the acid extract to 1 of base extract, 2 volumes of acid extract to 1 of base extract, and equal quantities of the two. This series was then diluted one to 200 and 0.25 cc. was injected. The individual basic and acidic extracts gave the usual responses, but the mixtures gave responses predominantly similar to the basic extract. This evidence gives great weight to the view that there is no inhibiting substance which is destroyed by the alkali as has been previously suggested (2) and refuted (5, 6).

DISCUSSION

It is our view that whatever the change, which would appear to be a cleavage or rearrangement, the phenomenon of potentiation is one which involves the native hormone molecule. Almost irrefutable evidence that the hormone molecule is directly and irreversibly altered by the higher pH values as a function of the concentration of hydroxyl ions is furnished by the following facts. *a*) The pressor substance is not present in the anterior lobe of the whale (3) although the melanophore hormone from this source can be potentiated.[2] Hence the presence of the pressor hormone is unessential for potentiation. *b*) The purified hormone can be potentiated (6). *c*) The acid extracted gland does not inactivate the potentiated material. *d*) The response is not quantitatively the same after alkali treatment and may indeed be qualitatively different (4, 6).

SUMMARY

There is a gradual potentiation of the melanophore hormone at pH values below 8. This potentiation becomes much more prominent at higher values.

There is no evidence that the pressor or any other substance present in the extract acts as a specific inhibitor of the melanophore hormone. Hence the view that potentiation is the result of the destruction of an inhibitor is untenable.

It is thought at present that potentiation of the hormone is due to an effect on the molecule, either rearrangement or cleavage due to changes in pH.

REFERENCES

1. GERLOUGH, T. D.: *J. Am. Chem. Soc.* 52: 825. 1930.
2. HOGBEN, L., AND C. GORDON: *J. Exper. Biol.* 7: 286. 1930.
3. OLDHAM, F. K., N. O. CALLOWAY AND E. M. K. GEILING: *Ergebn. d. Physiol.* In press.
4. CALLOWAY, N. O., R. M. MCCORMACK AND NERISSA P. SINGH: *Endocrinology* 30: 000. 1942.
5. JORES, A.: *Ztschr. f.d. ges. exper. Med.* 87: 266. 1933.
6. STEHLE, R. L.: *J. Pharmacol. & Exper. Therap.* 57: 1. 1936.
7. LANDGREBE, F. W., AND H. WARING: *Quart. J. Exper. Physiol.* 31: 31. 1941.

[2] Since our work was completed and the manuscript prepared, our attention has been called to recent work (7) which substantiates our viewpoint.

EFFECT OF PRESSOR AND OXYTOCIC FRACTIONS OF POSTERIOR PITUITARY EXTRACT ON THE LOSS OF WATER ADMINISTERED TO ALBINO RATS

ELDON M. BOYD AND NORMAN D. GARAND
From the Department of Pharmacology, Queen's University
KINGSTON, ONTARIO

DURING THE PAST TWENTY YEARS OR SO there has appeared variegated evidence which could be taken to suggest that in pharmacopoeial extracts of the posterior pituitary lobe there is present activity which is directed toward water balance in general rather than only toward the formation of urine. The evidence for a water-balance principle has been obtained most strikingly from experiments in frogs in which it is found that while posterior pituitary extract has a minor and fleeting effect upon urine formation, it influences total body water, as measured by changes in total body weight, to a pronounced degree. A reaction of this type in frogs is one which is referred to as the Brunn reaction and which consists of an uptake of water by frogs when injected with neurohypophyseal extract. Heller (1) has shown by assay that the proportion of what may be called the Brunn principle to the mammalian antidiuretic principle varies in extracts of the hypophysis obtained from different classes of the vertebrate subphylum. This evidence clearly suggests that the Brunn water-balance principle and the mammalian antidiuretic principle are not identical. Heller (1) further demonstrated in a similar manner that the Brunn principle was not identical with the oxytocic, pressor and melanophore fractions of posterior pituitary extract.

A Brunn reaction has been produced in many aquatic animals but so far its counterpart in birds and mammals has not been shown and it was with this end in view that the present investigation was undertaken.

A Brunn reaction has been produced in many amphibia including *Amblystoma* (2), *Necturus* (3), *Rana catesbiana* (4), *Rana clamitans* (3), *Rana esculenta* (5), *Rana pipiens* (4), *Rana septentrionalis* (4), *Rana temporaria* (6), *Leptodactylis ocellatus* (7), and *Bufo americanus* (3). Boyd and Dingwall (8) demonstrated a Brunn reaction in several reptiles including *Alligator mississippiensis*, *Chrysemys picta*, *Chyledra serpentina*, and *Zamenis constrictor*. Boyd and Dingwall (8) investigated several species of fish including *Notropis cornutis*, *Ictaluras lacustris*, *Perca flavescens*, *Eupomatis gibbosus* and *Ambloplites rupestris*, without being able to produce any Brunn reaction although acetic acid extracts of fish pituitaries would produce a Brunn reaction in frogs, a finding confirmed by Heller (1).

In birds and mammals, the effect of posterior pituitary extract has been measured upon water loss, rather than upon water uptake. In an attempt to bring the two types of experiment into line, Boyd and White (9) demonstrated that posterior pituitary extract inhibited the loss of body water by frogs kept out of water. As evidence that the latter reaction may be similar to that of the Brunn reaction, Boyd and White (9) reported that the duration of effect was similar in both reactions, that a similar dose of posterior pituitary extract produced both reactions and that oxytocic fractions were more effective, dose for dose, than pressor fractions of posterior pituitary extract in both reactions. In a later paper, Boyd and White

Received for publication December 26, 1941.

(10) found that posterior pituitary extract in similar doses and over a similar interval inhibited the loss of water injected into frogs and upon this reaction oxytocic fractions were also more effective than pressor fractions of the extract.

From the above considerations, it is apparent that several characteristics of the Brunn reaction appear in the effect of posterior pituitary extract on water loss in frogs and in particular may be noted the fact that oxytocic fractions were more effective than pressor fractions in all of these reactions. As an oxyocic fraction, pitocin (N.N.R.) was used in this work and as a pressor fraction, pitressin (N.N.R.). If mammalian body water reacts in a similar manner, then it should be possible to show that pitressin is less effective than pitocin in inhibiting the loss of total body water in mammals. Note that what is proposed is that the effect is on total body water, not upon urine formation, because it is well known that pitressin is more effective than pitocin as an antidiuretic in mammals.

To investigate this hypothesis, an assay of the total body-water-retaining effect of pitocin and pitressin was performed upon albino rats given water by mouth. The technique employed was similar to that given in a previous paper in which were described factors which affect the action of posterior pituitary extract on total body water in albino rats (11). Healthy, young albino rats weighing 125 to 200 gm. were starved and dehydrated overnight, weighed carefully, given by stomach tube 5 per cent of their body weight of distilled water and then re-weighed at intervals of 30 minutes for 6 hours. At the same time that water was given, pitocin or pitressin was injected subcutaneously in graded doses of from 10^{-6} to 20 cc. per kilogram of body weight, corresponding to from 5×10^{-6} to 100 mg. of pharmacopoeial standard posterior pituitary powder. Twelve animals were used with each dose and 12 controls received an equivalent volume of vehicle.

The mean changes in body weight in each group of 12 animals were calculated at each interval and expressed as per cent changes from the initial weight plus the weight of injected water. The amount of water retained by pitocin and pitressin was then calculated as a percentage of the amount lost by the controls. In these experiments, the total loss in body weight was taken as an index of the loss of body water which was not entirely correct because some loss of dry weight occurred in the stool and some was used up in body metabolism. The experiment corresponded, however, to similar experiments in frogs with which it was desired to compare the effect of pitocin and pitressin.

The results indicated that pitocin was not more effective than pitressin in causing retention of total body water in albino rats. It was not possible to confirm in mammals the corresponding reaction in frogs. Pitressin was effective in retaining body water in albino rats at doses as low as 5×10^{-3} cc. per kilogram of body weight while pitocin was effective down to only 5×10^{-2} cc. per kilogram. Pitressin was thus at least 10 times as effective as pitocin which corresponds to the partition of mammalian antidiuretic principle between the two fractions.

To summarize these results graphically, the mean percentage retention of total body water over a period of 5 hours was calculated for each dose of pitocin and pitressin. These values were then plotted against dosage and the results are shown in figure 1.

DISCUSSION

The Brunn reaction in frogs, named after F. Brunn who described it in 1921 (12), is an example of an effect of posterior pituitary extract upon body water apart from that upon urine formation. In 1930, Heller (13) showed that this reaction was produced more readily by oxytocic fractions of posterior pituitary extract, such as

orasthin and pitocin, than by pressor fractions such as tonephin and pitressin. This was confirmed by many investigators including Steggerda and Essex (14), Oldham (15) using postlobin-O and postlobin-V, Boyd and Brown (4) and Boyd and Mack (16). Boyd and White (9, 10) demonstrated that pitocin was also more effective than pitressin in causing retention of normal body water and of injected water in frogs. In the present communication it has been found that pitocin is not more effective than pitressin but rather less so, in retaining body water in a mammal, the albino

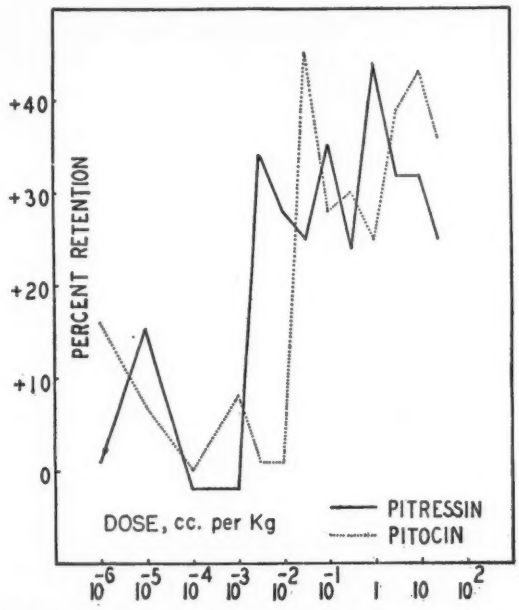


Fig. 1. Mean effect over a period of 5 hours of pitocin and pitressin on the percentage retention of total body water in albino rats given water by mouth.

rat. It has not been possible by this means to prove an effect upon mammalian body water, rather than upon mammalian urine formation, of posterior pituitary extract.

SUMMARY

Healthy, young, fasted and dehydrated, albino rats were given by mouth 5 per cent of their body weight of distilled water and varying doses of pitocin and pitressin, the effect upon total body water being measured as effect upon total body weight. Doses of pitressin of from 5×10^{-3} to 20 cc. per kg. of body weight had an identical effect and caused an average retention over 5 hours of about $\frac{1}{3}$ more water than in controls receiving no pitressin. Doses of pitocin of from 5×10^{-2} to 20 cc. per kg. of body weight had a corresponding effect. Pitressin was at least 10 times and not more than 50 times as effective as pitocin. These results do not conform to those of the corresponding experiment in frogs and indicate that in this respect at least, posterior pituitary extract does not have the same effect upon mammalian water balance as upon amphibian water balance.

The authors wish to acknowledge with thanks generous donations of pitocin and pitressin from Dr. E. A. Sharp of Parke, Davis and Company, Detroit, Michigan.

REFERENCES

1. Heller, H.: *J. Physiol.* 100: 125. 1941.
2. Bělehrádek, J., and J. S. Huxley: *Brit. J. Exper. Biol.* 5: 89. 1927.
3. Steggerda, F. R.: *Proc. Soc. Exper. Biol. & Med.* 36: 103. 1937.
4. Boyd, E. M., and G. M. Brown: *Am. J. Physiol.* 122: 191. 1938.
5. Granaat, D., and J. Hillesum: *Arch. néerl. de physiol.* 22: 347. 1937.
6. Rey, P.: *Comp. rend. Soc. de biol.* 118: 949. 1935.
7. Biasotti, A.: *Compt. rend. Soc. de biol.* 88: 361. 1923.
8. Boyd, E. M., and M. Dingwall: *J. Physiol.* 95: 501. 1939.
9. Boyd, E. M., and D. W. White: *Am. J. Physiol.* 124: 759. 1938.
10. Boyd, E. M., and D. W. White: *Am. J. Physiol.* 125: 415. 1939.
11. Boyd, E. M., and N. D. Garand: *Am. J. Physiol.* 130: 403. 1940.
12. Brunn, F.: *Ztschr. f. d. ges. exper. Med.* 25: 170. 1921.
13. Heller, H.: *Arch. f. exper. Path. u. Pharmakol.* 157: 323. 1930.
14. Steggerda, F. R., and H. E. Essex: *Proc. Soc. Exper. Biol. & Med.* 32: 425. 1934.
15. Oldham, F. K.: *Am. J. Physiol.* 115: 275. 1936.
16. Boyd, E. M., and E. G. Mack: *Proc. Soc. Exper. Biol. & Med.* 42: 70. 1939.

CORRELATIONS BETWEEN THE CHEMICAL STRUCTURE AND THE PHARMACOLOGICAL ACTIONS OF THE STEROIDS[1]

HANS SELYE

From the Department of Anatomy, McGill University
MONTREAL, CANADA

THE IMPORTANCE OF PHENANTHRENE derivatives in pharmacology and physiology is clearly shown by the fact that so many useful drugs and essential metabolites belong to this group. Morphine, codeine, colchicine, the bile acids, the digitalis aglucons, the toad poisons, many carcinogenic hydrocarbons, the vitamin D group, cholesterol and the steroid hormones all possess the phenanthrene or perhydrophenanthrene nucleus. Up to the present comparatively little attention has been given to the correlations which exist between the chemical structure and the pharmacological actions of the cyclopentano-perhydro-phenanthrene derivatives, and hence it was decided to make a systematic study of this field since an understanding of these correlations is a necessary prerequisite for any planned attempt to synthesize more potent compounds than are now available. Such an investigation was particularly tempting because the very important steroid hormones and their metabolites belong to this group. The recently discovered fact (137) that hormonally active steroids, if administered intraperitoneally or intravenously, produce anesthesia in the rat, supplies us with a simple test revealing pharmacological activity among such compounds. In the present communication we should like to summarize the most outstanding results of these investigations, which were possible only through the full-hearted cooperation of numerous outstanding steroid chemists of many countries, who furnished us with the necessary compounds.[2]

Although the main object of our study was to correlate chemical structure with the anesthetic effect of the steroids, in some cases special assays were performed to examine the compounds for other hormonal actions.

EXPERIMENTAL METHODS

Since it was found that the anesthetic effect of the steroids is increased by partial extirpation of the liver (138), apparently because they are normally detoxified in this organ, we performed our assays in the partially hepatectomized rat. In order to facilitate a comparative study, it appeared desirable to establish a R.U. of anesthetic effect. This anesthetic rat unit (A.R.U.) is defined as the minimum amount of a steroid which, when injected intraperitoneally, causes loss of the righting reflex in 4 of 6 female rats

Received for publication October 28, 1941.

[1] The expenses of this investigation were defrayed through a grant received from the Committee on Endocrinology of the United States National Research Council.

[2] Our thanks are due to Professors L. Ruzicka of the University of Zürich, L. Fieser, Harvard University, R. D. H. Heard, Dalhousie University, T. Reichstein, University of Basel, E. C. Kendall, Mayo Clinic, J. S. L. Browne and E. Venning, Royal Victoria Hospital, Montreal, M. Ehrenstein, University of Pennsylvania, and Doctors C. Scholz, of Ciba Pharmaceutic Products Inc., E. Schwenk, Schering Corporation, O. Kamm, Parke, Davis and Company, A. D. Odell, Charles E. Frosst and Company, O. Wintersteiner and H. E. Stavely, of the Squibb Institute and D. Beall and A. S. Cook, of Ayerst, Mckenna and Harrison Ltd.

weighing 40 to 60 gm. and partially hepatectomized (according to the method of Waelsch and Selye (158b) 2 to 4 hours before the test. It is important to administer the steroids in highly concentrated and consequently more active solutions because a large quantity of the solvent decreases the anesthetic potency by delaying absorption. It soon became evident that most of the steroids tend to form fairly stable oversaturated solutions if they are dissolved in 20 mg. per cc. of peanut oil by gentle heating and then cooled down to body temperature just before injection. Hence this technic was used whenever possible. Only in the case of very labile compounds is it preferable to take up the crystals in a small amount of alcohol or chloroform which is then mixed with the oil and later evaporated in vacuo. Particularly oil insoluble compounds had to be administered in propylene glycol, but since this solvent causes peritoneal irritation these bioassays are not very reliable.

In the case of the compounds which received a great deal of attention from other investigators we made no quantitative assays for the common steroid hormone actions. If, on the other hand, a compound hitherto not known to possess any hormone-like activity proved to possess appreciable anesthetic potency an effort was made to assay it for hormonal effects. The amount of material available was very small in the case of some compounds, but whenever possible bioassays were carried out for the 4 main types of hormonal activities, namely, folliculoid, corticoid, luteoid and testoid.[3]

The folliculoid activity of a compound was easily determined without loss of material since the bioassays for anesthetic potency were always performed on immature female rats. Opening of the vulvar orifice and histological signs of keratinization of the vagina, which was taken for section 24 hours after intraperitoneal injection of a compound, were regarded as positive evidence of folliculoid activity. It is of interest in this connection that steroids possessing a high degree of testoid, corticoid or luteoid activity usually elicited only temporary keratinization which was soon followed by mucification of the surface layer. Indeed, in many instances one part of the vagina was cornified, while another part was already mucified 24 hours after injection of these compounds. It is well known that small doses of typical folliculoids (estradiol, estrone) likewise cause mucification so that it appears probable that incidental factors determine whether the surface epithelium of the vagina responds with cornification or mucification. Since these tests were only qualitative they had to be completed, whenever sufficient material was available, by quantitative assays on ovariectomized immature rats receiving bidaily subcutaneous injections of the compound in oily solution.

The luteoid activity was tested on immature rabbits pretreated with estradiol by the McPhail test (94).

The testoid activity was assayed by the ability of a compound to stimulate the growth of the seminal vesicles and prostate in immature castrate male rats to which the steroid was given subcutaneously in oil twice daily.

The corticoid activity was assayed on immature adrenalectomized male rats which received bidaily subcutaneous injections of the compounds in oil. Only in the case of readily water-soluble steroids were we able to use the more rapid and sensitive bioassay method described by Selye and Schenker (144b).

Gonadotropic activity could be determined only in a few compounds since such assays require comparatively large amounts of the substance. This activity was assayed in hypophysectomized rats in which gonadotropic steroids maintain the sperma-

[3] These terms are used here in accordance with the recently proposed terminology of the steroid hormone actions (139) instead of the rather clumsy terms 'estrogenic' or 'follicular hormone-like,' 'adrenal cortical hormone-like,' 'corpus luteum hormone-like' or 'progestational' and 'testis hormone-like' or 'androgenic.'

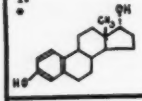
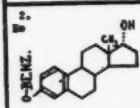
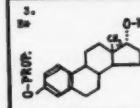
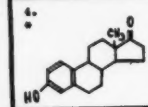
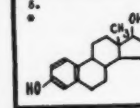
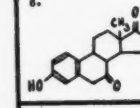
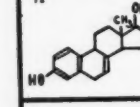
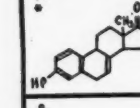
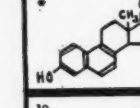
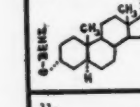
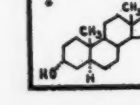
togenic epithelium in a normal condition and prevent testis atrophy. In some instances the ability of such compounds to prevent the testis atrophy normally produced by overdosage with folliculoids, was used as an indicator of activity.

EXPERIMENTAL RESULTS

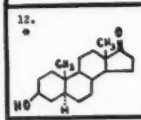
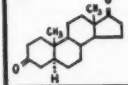
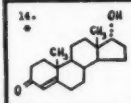
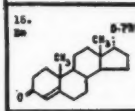
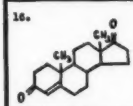
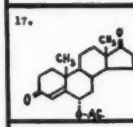
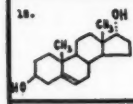
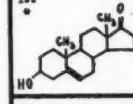
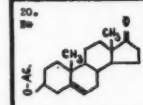
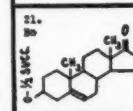
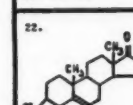
The results of the animal experiments with 75 steroid compounds are summarized in table 1. The steroids are arranged according to their chemical structure enumerating estrane, androstane, alkyl-substituted androstane, etiocholane and alkyl-substituted etiocholane derivatives in this order. Vitamin D_2 and stilbestrol which cannot readily be fitted into this scheme are added at the end of the list. Within each of these groups the individual compounds are arranged according to increasing numbers of C atoms. Among those having the same carbon skeleton the compounds are enumerated according to increasing degrees of unsaturation, these being subdivided again according to increasing degrees of oxygenation. An asterisk underneath the serial number of a compound indicates that its presence in animal tissue has been demonstrated. The esters of these naturally occurring compounds are marked 'E.' In the 1st column of the table are given the probable structural formulae of the compounds and in the 2nd column their systematic and (*in italics*) their popular names. The name of the chemist who supplied us with the compound is given in parentheses in each case. The structure of some steroids has not been completely proven as yet and in such instances we assigned to them the structure which is considered most probable on the basis of the information now at hand. In order to avoid any uncertainty concerning the identity of these compounds, the melting point of the sample used is given in the next column. In all instances these were determined in our laboratory with the help of a short stem melting point apparatus which requires no correction. We believe that it is indispensable to mention the full systematic name and give at least one physical characteristic (e.g. the melting point) of any compound used for pharmacolgoical studies, since a considerable amount of confusion has resulted in the past from the habit of referring to a substance merely by a corrupted or abbreviated name such as 'the diol of androsterone,' 'dehydro-androsterone' and 'pregnenediol.' Any one of these terms might be used to designate several compounds, so that the reader is unable to tell from a publication of this kind which steroid had been used and consequently the pharmacological investigation becomes valueless. The M.P. helps to estimate the approximate degree of purity of the sample used. Underneath the M.P. of our sample in brackets is given the correct M.P. of a highly purified preparation as described in the literature with a reference to the source from which this information was obtained. This reference may also help the interested reader to gain further information concerning the chemical properties of these compounds. In the next column is registered in mg. the amount of each compound which corresponds to one A.R.U. Whenever all dose levels tested proved devoid of anesthetic power the compound is marked 'inactive', giving in parentheses the highest dose level tested. In some cases the highest dose which it was possible to test caused some degree of narcosis, but did not suffice to produce the deep anesthesia required by the definition of 1 A.R.U. In these cases the highest dose tested was marked as less than 1 A.R.U.

In the following 4 columns are recorded the approximate corticoid (*C*), luteoid (*L*), folliculoid (*F*) and testoid (*T*) activities as judged by our own hitherto unpublished experiments and studies reported in the literature. We found it impossible to express the activity of each compound in a generally accepted unit since most of them were assayed in different laboratories and with the aid of different methods. Any attempt, for instance, to translate the 'mouse unit' of one worker into a 'rat unit' or 'capon unit' of another investigator would only be misleading. On the other hand it is indis-

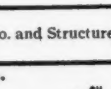
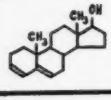
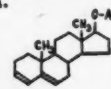
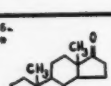
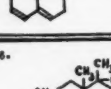
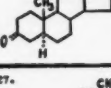
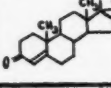
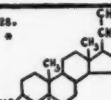
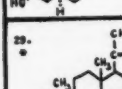
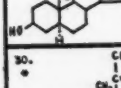
CORRELATION BETWEEN CHEMICAL STRUCTURE AND PHARMACOLOGICAL ACTION

No. and Structure	Name	M.P. °C.	A.R.U. in mg.	Hormonal Activity			
				C	L	F	T
1. 	$\Delta^{1,3,5}$-Estratriene-3,17(α)-diol	176 (176–177)	>20.0			3^{1}	$1?^{2}$
	α-Estradiol						
	(Schwenk)	23				15 80 145	76
2. 	$\Delta^{1,3,5}$-Estratriene-3, 17(α)-diol-3-benzoate	192–193 (193)	Inactive (20.0)		0	3^{1}	$0?^{2}$
	α-Estradiol benzoate						
	(Schwenk)	96			40	39	100
3. 	$\Delta^{1,3,5}$-Estratriene-3, 17(α)-diol-dipropionate	101–102 (104–105)	Inactive (20.0)			3^{1}	
	α-Estradiol-dipropionate						
	(Scholz)	97				39	
4. 	$\Delta^{1,3,5}$-Estratriene-3-ol-17-one	254–256 (256–259)	>20.0	0^{4}	0	3^{1}	$1?^{2,5}$
	Estrone						
	(Schwenk)	71		35	106 165	22 48	21 46 49 65
5. 	$\Delta^{1,3,5}$-Estratriene-3, 16, 17-triol	280–281 (282–283)	>20.0	0		$2–3^{1}$	
	Estriol						
	(Cook)	82		55		15 155	
6. 	$\Delta^{1,3,5}$-Estratriene-3-ol-7, 17-dione	211–214.5 (212–212.5)	>20.0			1^{1}	
	7-Keto estrone						
	(Fieser)	108				39	
7. 	$\Delta^{1,3,5,7}$-Estratetraene-3, 17(β)-diol	217–218	>20.0			1^{1}	
	17(β)-Dihydro equilin						
	(Wintersteiner)					39	
8. 	$\Delta^{1,3,5,7}$-Estratetraene-3-ol-17-one	238 (238–240)	Inactive (20.0)			3^{1}	
	Equilin						
	(Fieser)	53				22 29 39	
9. 	$\Delta^{1,3,5,6,8}$-Estrapentaene-3-ol-17-one	256 (257–258)	>20.0			2^{1}	0^{2}
	Equilenin						
	(Heard)	83				18 54 57	
10. 	Androstane-3(α), 17(α)-diol-3-benzoate	217–228	>10.0			1^{6}	
	Dihydroandrosterone benzoate *Andradiol benzoate*						
	(Schwenk)					134	
11. 	Androstane-3(α)-ol-17-one	177–178 (180–181)	4.5	0	0	$0–1^{6}$	3^{7}
	Androsterone *Cis-androsterone*					8 26	
	(Odell)	20		148 149	12 55	59 134	14 24

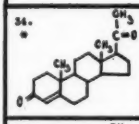
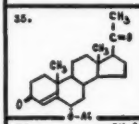
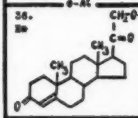
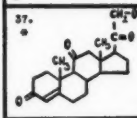
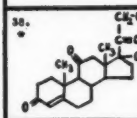
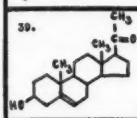
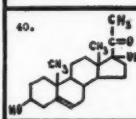
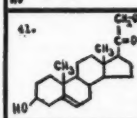
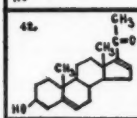
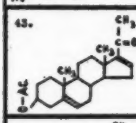
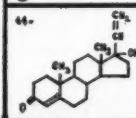
CORRELATION BETWEEN CHEMICAL STRUCTURE AND PHARMACOLOGICAL ACTION—*Continued*

No. and Structure	Name	M.P. °C.	A.R.U. in mg.	Hormonal Activity			
				C	L	F	T
12. 	Androstane-3(β)-ol-17-one	170.5–171.5 (174–175)	4.5				2
	Iso-androsterone trans-androsterone						
	(Fieser)	120					14 24 120
13. 	Androstane-3, 17-dione	132–134 (132–133)	7.5			1[7,8]	3
	(Heard)	5				26	11 121 129 156
14. 	Δ[4]-Androstene-3-one-17(α)-ol	154 (154–154.5)	7.0[9]	0[4]	0–2	1[6]	3
	Testosterone					42	
	(Schwenk)	19		53 133	55 70	104 119 141	121 128 130
15. 	Δ[4]-Androstene-3-one-17(α)-ol-propionate	118 (121)	20.0	0		1[6,8]	3
	Testosterone propionate						
	(Scholz)	131		109 149 160		1 74	95 98 107
16. 	Δ[4]-Androstene-3, 17-dione	169–171 (172–174)	10.0[9]	0	0–1[10]	1[11]	3
	Androstenedione					4	8
	(Scholz)	114		149 160	55 70	8 42 73	121 129 156
17. 	Δ[4]-Androstene-3, 17-dione-6(α)-ol acetate	176 (176)	<5.0[12]			1[1]	
	(Ehrenstein)	32				134	
18. 	Δ[5]-Androstene-3(β), 17(α)-diol	182–183 (182–183)	>20.0			1[11]	3
	Androstenediol					3,4 26	
	(Schwenk)	125				47 134	126 134
19. 	Δ[5]-Androstene-3(β)-ol-17-one	137 (144–146)	Inactive (20.0)			0–1[11]	2
	Dehydro-iso-androsterone trans-dehydroandrosterone					27, 72 75	24
	(Schwenk)	115				105 154 157	72 76 129
20. 	Δ[5]-Androstene-3(β)-ol-17-one acetate	169 (169)	Inactive (20.0)			1[1]	2
	Dehydro-iso-androsterone acetate						
	(Schwenk)	162				134	134
21. 	Δ[5]-Androstene-3(β)-ol-17-one half succinate	255–257	7.0				
	Dehydro-iso-androsterone half succinate						
	(Fieser)						
22. 	Δ[5]-Androstene-3-chloro-17-one	155–156	Inactive (20.0)				
	3-Chloro-dehydro-iso-androsterone						
	(Fieser)						

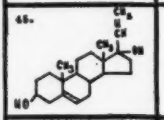
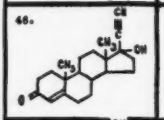
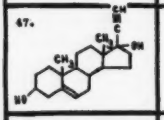
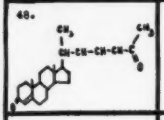
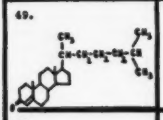
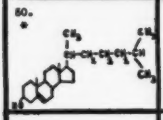
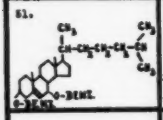
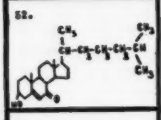
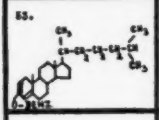
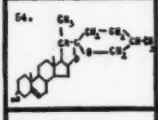
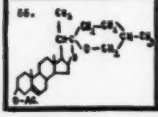
CORRELATION BETWEEN CHEMICAL STRUCTURE AND PHARMACOLOGICAL ACTION—*Continued*

No. and Structure	Name	M.P. °C.	A.R.U. in mg.	Hormonal Activity			
				C	L	F	T
25.	$\Delta^{5,6}$-Androstadiene-17-ol	141–143 (146)	12.0[3]				1
	(Fieser)	7					7
26. 	$\Delta^{5,6}$-Androstadiene-17-ol acetate	120–122 (147.4)	Inactive[13] (15.0)				
	(Fieser)	7					
25. * 	$\Delta^{5,6}$-Androstadiene-17-one	91–93 (87–88)	Inactive[13] (15.0)	0		0	2
	(Fieser)	2		2		2	7
26. 	17-Methylandrostane-3-one-17-ol	193–194 (192–193)	6.0		2	1[11]	3
	Methyl-dihydro-testosterone						24 121
	(Ruzicka)	121			70	26 163	128 163
27. 	17-Methyl-Δ^4-androstene-3-one-17-ol	153 (163–164)	8.0[9]			0–1[6]	3
	Methyl testosterone						24 25 28 128
	(Schwenk)	121				47 163	
28. * 	17-Ethyl-androstane-3(β), 20(β)-diol	196 (195–196)	>20.0	0		0	
	Allo-pregnanediol						
	(Heard)	85		160		134	
29. * 	17-Ethyl-androstane-3(β)-ol-20 one	190–192 (193–194)	12.0[14]		0	1	
	Allo-pregnanolone Wintersteiner's cpd. 'A'						
	(Beall)	86			12 55	31[15] 134	
30. * 	17-Ethyl-androstane-3, 20-dione	195–197 (199–200)	10.0[14]			0[15]	
	Allo-pregnanedione						
	(Beall)	85				31 134	
31. 	17-Ethyl-androstane-3(β), 17, 20-triol-3,20-diacetate	159–161 (159–161)	Inactive[16] (30.0)	0			
	Reichstein's cpd. 'J'-diacetate						
	(Reichstein)	110		113			
32. 	17-Ethyl-androstane-3(β), 17-diol-20-one-3 acetate	172 (191)	Inactive[17] (10.0)	0			
	Reichstein's cpd. 'L'-acetate Wintersteiner's cpd. 'G'-acetate						
	(Kendall)	38		161			
33. * 	17-Ethyl-androstane-3(β), 21-diol-11, 20-dione	139 (189–191)	7.0	0			
	Kendall's cpd. 'H' Reichstein's cpd. 'N/8'						
	(Kendall)	152		67 92			

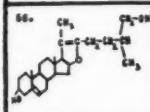
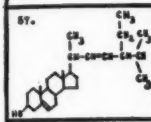
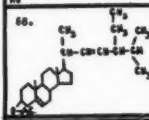
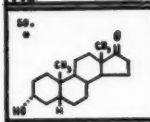
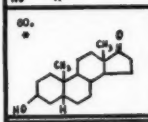
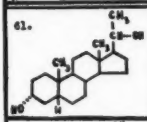
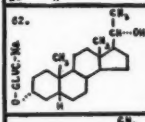
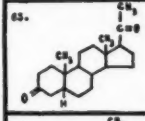
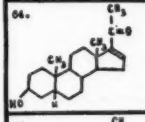
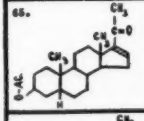
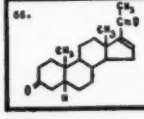
CORRELATION BETWEEN CHEMICAL STRUCTURE AND PHARMACOLOGICAL ACTION—*Continued*

No. and Structure	Name	M.P. °C.	A.R.U. in mg.	Hormonal Activity			
				C	L	F	T
34. 	17-Ethyl-Δ^4-androstene-3, 20-dione	128 (128)	2.0	2	3	1[8]	0–1
	Progesterone			16. 17 34 43 51 151	9 12 55 118	134[a]	
	(Schwenk)	146					
35. 	17-Ethyl-Δ^4-androstene-3, 20-dione-6(α)-ol-acetate	145–146 (145–146)	<5.0	1?	2	1?[12]	
	6-Hydroxy-progesterone acetate						
	(Ehrenstein)	33		33	33	134	
36. 	17-Ethyl-Δ^4-androstene-3, 20-dione-21-ol acetate	152 (158–160)	1.0	3[18]	2	0–1[8]	0–1
	Desoxycorticosterone acetate *Kendall's cpd. 'B' acetate* *21-hydroxy-progesterone*			68 69 151	56 58 60 81	99 103 134	99 102
	(Schwenk)	115		164	134	134b	134
37. 	17-Ethyl-Δ^4-androstene-3, 11, 20-trione-21-ol	173 (174–180)	3.5	3[19]			
	Dehydrocorticosterone *Kendall's cpd. 'A'* *Reichstein's cpd. '8'*			62 69 78 92			
	(Kendall)	116					
38. 	17-Ethyl-Δ^4-androstene-3, 11, 20-trione-17, 21-diol	215–218 dec. (215–218 dec.)	15.0	0–2		1	0
	Kendall's cpd. 'E' *Reichstein's cpd. 'FA 6'* *Wintersteiner's cpd. 'F'*			78 91 111 112 161		134	93
	(Kendall)	78					
39. 	17-Ethyl-Δ^5-androstene-3(β)-ol-20-one	186 (185–187)	12.0	1		1	1?
	Pregnenolone						
	(Schwenk)	6		135		134	134
40. 	17-Ethyl-Δ^5-androstene-3(β), 17-diol-20-one	174–176	10.0	0		1	0
	Δ^5-Pregnene-3(β)-17-diol-20-one						
	(Stavely)			134		134	134
41. 	17-Ethyl-Δ^5-androstene-3(β), 21-diol-20-one-21-acetate	183–184 (183–184)	4.0	2		1	0
	Aceloxypregnenolone			61 135			
	(Schwenk)	61		159		134	134
42. 	17-Ethyl $\Delta^{5,16}$-androstadiene-3(β)-ol-20-one	205–210 (213–215)	>20.0			0	
	$\Delta^{5,16}$-Pregnadienolone						
	(Kamm)	88				134	
43. 	17-Ethyl-$\Delta^{5,16}$-androstadiene-3(β)-ol-20-one-acetate	174–175 (176)	Inactive (20.0)			0	
	$\Delta^{5,16}$-Pregnadienolone acetate						
	(Kamm)	10				134	
44. 	17-Vinyl-Δ^4-androstene-3-one-17-ol	139 (140–141)	10.0[9]			1?	
	Vinyl-testosterone						
	(Scholz)	123				134	

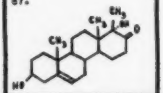
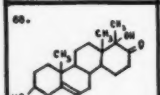
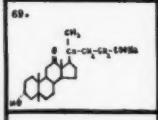
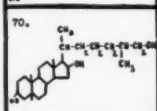
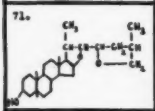
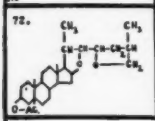
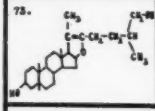
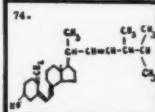
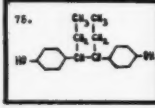
CORRELATION BETWEEN CHEMICAL STRUCTURE AND PHARMACOLOGICAL ACTION—*Continued*

No. and Structure	Name	M.P. °C.	A.R.U. in mg.	Hormonal Activity			
				C	L	F	T
45. 	17-Vinyl-Δ^5-androstene-3(β), 17-diol	185–186 (184–187)	10.0[9]			1	
	Vinyl-androstenediol (Scholz)	123				134	
46. 	17-Ethinyl-Δ^4-androstene-3-one-17(α)-ol	265–268 (270–272)	20.0	0	3	1[8]	0–1[10]
	Ethinyl-testosterone *pregneninolone* *Anhydro-hydroxy-progesterone* (Schwenk)	63		50 62	124 132 147	36 37 103 141	64
47. 	17-Ethinyl-Δ^5-androstene-3(β), 17(α)-diol	240 (240–242)	>20.0			1	
	Ethinyl-androstenediol (Stavely)	122				66	
48. 	17-*Iso*-heptyl-Δ^4-androstene-3, 25-dione	114–116	Inactive (40.0)			0	
	Δ^4-*Nor-cholestene-3, 25-dione* (Schwenk)					134	
49. 	17-*Iso*-octyl-Δ^4-androstene-3-one	80.5	Inactive (40.0)			0	
	Cholestenone (Schwenk)					134	
50. 	17-*Iso*-octyl-Δ^5-androstene-3(β)-ol	140 (150)	Inactive (40.0)	0–1[21]		0–1[21]	
	Cholesterol (Merck & Co.)	41		77 79 134 153		45 134	
51. 	17-*Iso*-octyl-Δ^5-androstene-3(β), 7(α)-diol dibenzoate	171–172	Inactive (20.0)			0	
	7(α)-*Hydroxy-cholesterol dibenzoate* (Wintersteiner)					134	
52. 	17-*Iso*-octyl-Δ^5-androstene 3(β)-ol-7-one	171–173	Inactive (25.0)			0	
	7-Keto-cholesterol (Wintersteiner)					134	
53. 	17-*Iso*-octyl-$\Delta^{3,4}$-androstadiene 3-ol-benzoate	121	Inactive (20.0)			0	
	Δ^4-*Cholestenone-enol-benzoate* (Scholz)					134	
54. 	*Diosgenin* (Kamm)	205–207	Inactive (20.0)			0	
						134	
55. 	*Diosgenin acetate* (Kamm)	196–198	Inactive (20.0)			0	
						134	

CORRELATION BETWEEN CHEMICAL STRUCTURE AND PHARMACOLOGICAL ACTION—*Continued*

No. and Structure	Name	M.P. °C.	A.R.U. in mg.	Hormonal Activity			
				C	L	F	T
56. 		162–174	Inactive (20.0)			0	
	Pseudo-diosgenin (Kamm)					134	
57. 	17-*Iso*-decyl-Δ^5-androstene 3(β)-ol	168–169 (170)	Inactive (40.0)			0	
	Stigmasterol (Schwenk)	41				134	
58. 	17-*Iso*-decyl-Δ^5-androstene 3(β)-ol-acetate	140	Inactive (40.0)			0	
	Stigmasterol acetate (Schwenk)					134	
59. 	Etiocholane-3(α)-ol-17-one	139–140 (148–150)	12.0			1?	
	3(α)-Etiocholanolone (Fieser)	13				134	
60. 	Etiocholane-3(β)-ol-17-one	151–153 (170)	12.0				
	3(β)-Etiocholanolone (Kamm)	30					
61. 	17-Ethyl-etiocholane-3(α), 20(α)-diol	237–238 (237–239)	>20.0[22]	0	0	0[15]	
	Pregnanediol (Cook)	87		160	12 44 55	44 90 134	
62. 	17-Ethyl-etiocholane-3(α), 20(α)-diol Na glucuronidate	271 (271)	>20.0			0	
	Pregnanediol sodium glucuronidate (Browne and Venning)	158				134	
63. 	17-Ethyl-etiocholane-3, 20-dione	121 (120)	0.8	0	0	0	0
	Pregnanedione (Beall)	101		134	12 55	134	134
64. 	17-Ethyl-Δ^{16}-etiocholane-3(β)-ol-20-one	188 (188–190)					
	Δ^{16}-Pregnenolone (Kamm)	84					
65. 	17-Ethyl-Δ^{16}-etiocholane-3(β)-ol-20-one acetate	141–143	Inactive (20.0)			1	
	Δ^{16}-Pregnenolone acetate (Kamm)					134	
66. 	17-Ethyl-Δ^{16}-etiocholene-3, 20-dione	190 (200–201)	>20.0			0	
	Δ^{16}-Pregnenedione (Kamm)	89				134	

CORRELATION BETWEEN CHEMICAL STRUCTURE AND PHARMACOLOGICAL ACTION—*Continued*

No. and Structure	Name	M.P. °C.	A.R.U. in mg.	Hormonal Activity			
				C	L	F	T
67. 	17$^{\alpha}$-Methyl-chrysopregnene-3(β), 17$^{\alpha}$-diol-17-one	180–182	5.0			1?	
	(Stavely)					134	
68. 	17$^{\alpha}$-Epimer of above	275–278 (276–278)	5.0			1	
	(Stavely)	150				134	
69. 	*3-Hydroxy-12-keto-sodium cholanate*		Inactive (20.0)			0	
	(Odell)					134	
70. 	*Tetrahydro sarsasapogenin*	190–191	Inactive (20.0)			0	
	(Kamm)					134	
71. 	*Sarsasapogenin*	198–200	Inactive (20.0)			0	
	(Kamm)					134	
72. 	*Sarsasapogenin acetate*	144–146	Inactive (20.0)			0	
	(Kamm)					134	
73. 	*Pseudo-sarsasapogenin*	164–170	Inactive (20.0)				
	(Kamm)						
74. 	*Calciferol Vitamin D*[2]	116 (116)	Inactive (30.0)			0	
	(Odell)	41				134	
75. 	4: 4[1]-Dihydroxy-α: β-diethylstilbene	166	20.0	0[4]		3	
	Stilbestrol			144		15	

[1] Mainly cornifying. [2] Slightly stimulates certain parts of the penis in castrate rat. [3] No. effect on capon's comb. [4] Toxic for adrenalectomized rats. [5] Stimulated muscle and fibrous tissue of prostate and seminal vesicles. [6] Mainly mucifying. [7] Inactivity established only for testoid urine extracts which probably contained androsterone.
[8] Metrotropic in immature rat. [9] Produced convulsions. [10] Inactive for guinea pig receptivity; active on rabbit endometrium. [11] Some authors claim it causes vaginal cornification while others say it inhibits or mucifies. [12] Anesthesia terminated fatally.
[13] Tested on 1 rat only. [14] Prolonged anesthesia. [15] Stimulates ovopositor in bitterling. [16] Tested on 40-mg. non-hepatectomized rat. [17] Tested on 3 rats.
[18] Mainly salt metabolism active. [19] Mainly carbohydrate metabolism active.
[20] Stimulates clitoris and female prostate. [21] Contrary to our experiments.
[22] One A.R.U. 20.0 mg. when given in oil; in propylene glycol, 1 A.R.U. = 5 mg.

pensable, for studies concerning the correlation between chemical structure and pharmacological activity, to have some idea concerning the approximate potency with regard to the various hormonal effects. Hence it was decided to adopt an arbitrary scale ranging from 0 to 3. In this scale 0 means entirely devoid of activity in all doses tested; 1 means that the compound exhibits only a trace of activity; 2 indicates marked activity, while grade 3 is given only to the compounds which belong to the most active substances of the corresponding series. Whenever the literature concerning the actions of a compound is very contradictory the extreme statements are recorded (e.g. 0 to 2 if one author reports no activity and another finds the compound markedly active). A blank means that the compound has not been assayed for the particular action by us, nor were we able to find any reference to such assays in the world literature. In the case of compounds which have frequently been assayed for a certain action (for instance the folliculoid activity of estradiol or the testoid activity of testosterone) only a few key references were selected which may help the reader to find more precise information concerning such bioassays. In the case of compounds about whose hormonal activity little is known as yet, all available references are given underneath the grade assigned to them in the scale of 0 to 3. Since in many cases these references are contained in foreign chemical journals not generally consulted by the pharmacologist, it was felt that they may prove useful to those who wish to consult these tables as a reference source. Reference numbers are given in bold face type.

Incidental data are given in footnotes to the table. For example, the solubility of the compounds in a concentration of 20 mg. per cc. in peanut oil at body temperature as they were injected for anesthesia tests. Unusual hormonal effects, the number of animals whenever less than 6 were used for the dose level considered to be 1 A.R.U., etc., are also reported in the footnotes.

In addition to these observations a particularly detailed study of the gonadotropic action of pregnenolone (*compound* 39) was made which proved highly effective in preventing the testicular atrophy normally caused by hypophysectomy in the rat. This observation is of special interest since previous work (105) appeared to indicate that only testoid substances are endowed with this action. Pregnenolone, however, is practically inactive as a testoid when tested on castrate male rats. It was even more surprising to note that although pregnenolone is highly effective in causing continuous vaginal cornification in spayed rats it inhibits the testicular atrophy normally caused by folliculoids (estradiol, estrone) in the intact animal (140b).

DISCUSSION

A survey of the experiments indicates that it is necessary to differentiate between merely subordinated and independent pharmacological actions among the steroids. It is well known for instance, that a testoid compound capable of stimulating the seminal vesicles will, merely by virtue of its testoid power, also stimulate other male accessory sex organs such as the preputial glands, the prostate, the penis, and the capon's comb. The kidney is not a male accessory sex organ, yet it is stimulated by various steroids in proportion to their testoid activity (140). Hence it is highly probable that the renotropic effect of steroids is merely subordinated to their testoid activity. Similarly, all folliculoid compounds cause vaginal cornification, enlargement of the uterus and breasts and hypertrophy of the adrenal cortex, so that these actions must be regarded as subordinated to the folliculoid activity.

On the other hand, our experiments indicate that there are at least six independent or main pharmacological actions, namely, the folliculoid, corticoid, luteoid, testoid, gonadotropic and anesthetic effects. It is quite possible that further research will reveal the existence of additional independent pharmacological groups, but the above

mentioned 6 series have at this time already been shown to be of such character. The proof for this is that any one of these activities may be exhibited by a compound irrespective of any other hormonal properties which it may or may not possess. This has been proven sufficiently for the folliculoid, testoid, luteoid and corticoid activities and the experiments recorded in this communication show that the same is true of the anesthetic and gonadotropic effects. Pregnanedione (*compound* 63), which has the highest anesthetic power of all of the 75 steroids examined, is entirely devoid of corticoid, luteoid and testoid activities as shown by our bioassays and exhibits only a questionable trace of folliculoid potency. Consequently it appears justified to conclude that the anesthetic effect is independent of the last mentioned 4 hormonal actions. Similarly, the gonadotropic action of pregnenolone (*compound* 39) cannot be regarded as merely subordinated to its very weak testoid action since it has been shown (136, 142) that small doses of testoid compounds, far from protecting the testis from atrophy, actually decrease testicular size.

Another interesting fact concerning pregnenolone (*compound* 39) is that although it causes continuous vaginal cornification in the ovariectomized rat (in a dose of 250 μg. bidaily) it readily inhibits the testicular atrophy otherwise produced by estradiol in this species. This observation indicates that the structure of the pregnenolone molecule is such that it endows the compound with definite folliculoid activity (tested on the vaginal epithelium) and at the same time with the power to inhibit the action of folliculoids (tested on the testis). The fact that the same molecule may be endowed with two antagonistic pharmacological properties makes one wonder if the potential folliculoid power of a compound such as pregnenolone is not perhaps much greater than would appear from bioassays, since part of its own folliculoid effect may also be inhibited by its power to neutralize this same action. This problem appears of some theoretical importance because if similar conditions should exist among other steroids, the apparent action of such compounds would have to be regarded as the sum of their different antagonistic, and in some instances perhaps also synergistic properties.

Our findings indicate furthermore, that contrary to common belief an α-β unsaturated ketone group in position C_3 is not essential for corticoid activity. Both pregnenolone (*compound* 29) and acetoxy pregnenolone (*compound* 41) possess corticoid activity and yet they have neither a double bond in position C_{4-5} nor a ketone group in position C_3.

All steroids having a side chain of two or less C atoms exhibited some pharmacological action, while the long side chain derivatives were inert. This pharmacological action was in some cases a typical hormone effect, in other instances an anesthetic activity, while all of the long side chain derivatives were devoid of all these actions. An apparent exception to this rule are Reichsteins' compounds 'J' diacetate and 'L' acetate (*compounds* 31, 32) but there was not sufficient amounts of these to test them adequately. A careful perusal of the literature revealed only one exception to the above mentioned rule. This is the observation of Ruzicka et al. (127) who state that nor-cholestane-3(β)-ol-25-one, which has a long side chain, is very slightly testoid inasmuch as it contains one I.C.U. in 3 mg. Since the compound was not available we could not check this statement which has not been confirmed up to the present by other investigators.

The most common pharmacological actions were the anesthetic and folliculoid activities. It must be borne in mind that vaginal cornification and vaginal mucification are both characteristic of the action of folliculoids inasmuch as even the typical folliculoid substances (estradiol or estrone) cause mucification under certain circumstances as mentioned above. Among the substances examined only pregnenolone (*compound* 39) and androstenediol (*compound* 18) and the estrane derivatives (*compounds* 1–9)

were definitely proven to cause continuous vaginal cornification in chronic experiments. However, most of the other compounds, even though mainly mucifying in their action, caused transitory cornification of the vaginal epithelium. In this sense all steroids examined for this action showed some degree of folliculoid activity as long as they have no side chain or 1 of no more than 2 carbon atoms. Only in the case of pregnanediol (*compound 61*), pregnanedione (*compound 63*) and *allo*-pregnanediol (*compound 28*) were the vaginal changes so slight (moderate degree of cell proliferation) that the folliculoid effect remains doubtful. In general one may say, however, just as for the anesthetic activity, that no great specialization of molecular structure is required to produce the folliculoid effect. The fact that the ovipositor of the bitterling and the uterus of mammals are also readily stimulated by all types of steroid hormones gives further evidence in favor of the conception that the derivatives of the Müllerian ducts are highly sensitive to various short side chain steroids and are more or less nonspecific indicators of hormonal activity.

Esterification, which is known to alter the degree of hormonal activity, also exerts a marked influence on anesthetic potency. This is clearly indicated by the fact that though estradiol (*compound 1*) is distinctly active, its benzoate (*compound 2*) and dipropionate (*compound 3*) are inactive in the doses tested. Probably these esters are more slowly absorbed, a circumstance which increases their folliculoid power, but decreases their anesthetic effect. On the other hand, dehydroisoandrosterone (*compound 19*) and its acetate (*compound 20*) proved inactive in the doses used, presumably because they are comparatively insoluble, while the half succinate (*compound 21*) which is more soluble and consequently more capable of being rapidly taken up into the blood in a high concentration, exhibits a marked anesthetic potency.

The highest anesthetic effect is exhibited by compounds oxygenated only at the two extreme ends of the molecule, that is at C_3 and C_{17} among the steroids with no side chain and at C_3 and C_{20} or C_{21} among those having an alkyl group at C_{17}. Further oxidation is usually detrimental. Thus progesterone (*compound 34*) is more active than 6-hydroxy-progesterone (*compound 35*); desoxycorticosterone acetate (*compound 36*) is more active than dehydrocorticosterone (*compound 37*) which in turn is more active than Kendall's compound 'E' (*compound 38*). Oxygenation in a long side chain farther away from ring *D* than C_{21} does not endow a compound with anesthetic or hormonal activity as shown by *nor*-cholestenedione (*compound 48*) and the sarsasapogenins (*compounds 71, 72, 73*). In this connection it is important to realize that none of the long side chain steroids were oxygenated between C_{17} and C_{20}. The absence of oxygen at this pole of the steroid nucleus may equally well be responsible for their inactivity as the presence of a long side chain.

One double bond does not appear to interfere seriously with the anesthetic effect if it is situated in ring *A* or *B*, but two or more double bonds in these two rings or 1 double bond in ring *D* are detrimental. This is shown for instance by the fact that the fully saturated pregnanedione (*compound 63*) is more active than progesterone (*compound 34*) but the latter substance with its single double bond in ring *A* is still among the most active anesthetic compounds. On the other hand $\Delta^{3,5}$-androstadienes (*compounds 23, 24, 25*) which possess 1 double bond in ring *A* and 1 in ring *B* are all weakly active or inactive, while the estratrienes, estratetraenes and the estrapentaene (*compounds 1–9*) all of which possess 3 or more double bonds in the first 2 rings, are among the least active compounds of this series. In the case of the above mentioned dienes it must be kept in mind that the absence of oxygen at position C_3 may also have had something to do with their inactivity.

The steric position of a hydroxyl group at C_3 appears to be without importance for the anesthetic effect since both stereoisomeric etiocholanolones (*compounds 59*

and 60) are equally active and the same is true of the isomeric androsterones (*compounds 11* and *12*). Further investigations will have to show whether this rule also holds in the case of isomeric C_3 alcohols of the pregnane and *allo*-pregnane series.

A 5-membered ring *D* is not essential for the anesthetic effect since the two chrysopregnene derivatives examined (*compounds 67 and 68*) were both highly active.

It is rather surprising that stilbestrol (*compound 75*) which does not possess the phenanthrene nucleus also has some anesthetic effect. This observation is just as difficult to explain as the fact that this stilbene derivative is strongly folliculoid. Frequently when pharmacologists attempted to study the correlations between the chemical structure and the biological action of a series of related compounds, they were discouraged by similar instances which appeared to contradict their main conclusions. It must be kept in mind, however, that correlations established for one particular chemical series must not necessarily hold for another series. Obviously the barbiturates, and the ethers of aliphatic alcohols also possess anesthetic effects. Although definite structural prerequisites for this action have been established for each of these series, such rules are not transferable from one group to another. Similarly the correlation between structure and action will have to be studied separately in the stilbene series. At this time we are only beginning to uncover a few rules concerning such correlations and the fact that these are limited in their applicability, should not discourage research along these lines as it is evident that any systematic effort to prepare new compounds with certain specific pharmacological properties must be based on this type of study unless one is satisfied to rely merely on chance.

SUMMARY AND CONCLUSIONS

Seventy-five steroid compounds have been studied for their anesthetic effect in the rat. In many instances, additional bioassays for various hormonal activities have also been performed. These investigations led to the following main conclusions.

A distinction must be made between the subordinated and the independent actions of the steroids. The former are merely side effects of another pharmacological action with which they are invariably associated, as for instance the prostate-stimulating action of steroids is subordinated to their general testoid activity. However, 6 of the steroid hormone actions may be regarded as independent of each other. These are the folliculoid, corticoid, luteoid, testoid, gonadotropic and anesthetic effects.

Certain pharmacological actions, especially the anesthetic and vagina-stimulating effects are common to the great majority if not to all steroids having no side chain or one which consists of no more than 2 carbon atoms.

The highest anesthetic effect is exhibited by steroids oxygenated only at the two extreme ends of the molecule.

One double bond does not appear to interfere seriously with the anesthetic effect if it is situated in ring *A* or *B*, but 2 or more double bonds in these 2 rings or 1 double bond in ring *D* are detrimental.

Esterification decreases the anesthetic effect if it delays absorption, while the reverse is true in the case of particularly readily soluble esters.

The steric position of the hydroxyl group in position C_3 appears to be without importance for the anesthetic effect at least in the androstane and etiocholane series.

A 5-membered ring *D* is not indispensable either for the anesthetic or the folliculoid effect as shown by the 2 chrysopregnene derivatives in this series.

Contrary to common belief, an α-β-unsaturated ketone group in position C_3 is not essential for corticoid activity.

The same molecule may be endowed with 2 antagonistic pharmacological properties which can be demonstrated by the use of different test objects; for example, preg-

nenolone is folliculoid in the spayed rat inasmuch as it causes vaginal cornification yet it antagonizes the testis-atrophy-producing effect of estradiol another folliculoid compound.

The presence of a hydrogen at C_5, in a position *cis* to the C_{10} methyl group, (etiocholanes and 17-alkyl-etiocholanes) is incompatible with any definite, known hormonal activity but may be associated with high anesthetic potency.

REFERENCES

1. Aschheim, S., and J. Varangot: C. r. Soc. Biol. 130: 830. 1939.
2. Burrows, H., J. W. Cook, E. M. F. Roe and F. L. Warren. *Biochem. J.* 31: 950. 1937.
3. Butenandt, A.: *Die Naturwiss.* 24: 15. 1936.
4. Butenandt, A.: *Bull. Soc. chim. biol.* 19: 1477. 1937.
5. Butenandt, A., and H. Cobler: *Ztschr. f. physiol. chem.* 234: 218. 1935.
6. Butenandt, A., and W. Grosse: *Ber. Dtsch. Chem. Ges.* 70: 1446. 1937.
7. Butenandt, A., and A. Heusner: *Ber. Dtsch. Chem. Ges.* 71: 198. 1938.
8. Butenandt, A., and H. Kudszus: *Ztschr. f. physiol. Chem.* 237: 75. 1935.
9. Butenandt, A., and J. Schmidt: *Ber. Dtsch. Chem. Ges.* 67: 2088. 1934.
10. Butenandt, A., and J. Schmidt-Thomé: *Ber. Dtsch. Chem. Ges.* 72: 182. 1939.
11. Butenandt, A., and K. Tscherning: *Ztschr. f. physiol. Chem.* 229: 185. 1934.
12. Butenandt, A., U. Westphal and W. Hohlweg: *Ztschr. f. physiol. Chem.* 227: 84. 1934.
13. Butler, G. C., and G. F. Marrian: *J. Biol. Chem.* 124: 237. 1938.
14. Callow, R. K., and R. Deanesly: *Biochem. J.* 29: 1424. 1935.
15. Castrodale, D., O. Bierbaum, E. B. Helwig and C. M. MacBryde: *Endocrinology* 29: 363. 1941.
16. Corey, E. L.: *Proc. Soc. Exper. Biol. & Med.* 41: 397. 1939.
17. Corey, E. L.: *Proc. Am. Physiol. Soc.* 52nd Annual Meet. New Orleans, Louisiana, March, 1940.
18. David, K.: *Acta brev. Neerland.* 4: 63. 1934.
19. David, K.: *Acta brev. Neerland.* 5: 1. 1935.
20. David, K., and J. Freud: *Acta brev. Neerland.* 5: 31. 1935.
21. David, K., J. Freud and S. E. de Jongh: *Biochem. J.* 28: 1360. 1934.
22. David, K., and E. de Jongh: *Biochem. J.* 29: 371. 1935.
23. David, K., S. E. de Jongh and E. Laqueur: *Arch. Internat. de pharmacodyn. et de thérap.* 51: 137. 1935.
24. Deanesly, R., and A. S. Parkes: *Biochem. J.* 30: 291. 1936.
25. Deanesly, R., and A. S. Parkes: *Brit. Med. J.* 1: 527. 1936.
26. Deanesly, R., and A. S. Parkes: *Brit. Med. J.* 1: 257. 1936.
27. Deanesly, R., and A. S. Parkes: *Quart. J. Exper. Physiol.* 26: 393. 1937.
28. Dessau, F.: Acta brev. Neerland. 7: 1. 1937.
29. Dirscherl, W., and F. Hanusch: *Ztschr. f. physiol. Chem.* 233: 13. 1935.
30. Dorfman, R. I.: *Proc. Soc. Exper. Biol. & Med.* 46: 351. 1941.
31. Duyvené de Wit, J. J.: *Endocrinology* 24: 580. 1939.
32. Ehrenstein, M.: *J. of Organic Chem.* 6: 626. 1941.
33. Ehrenstein, M., and T. O. Stevens: *J. of Organic Chem.* 5: 318. 1940.
34. Emery, F. E., and P. A. Greco: *Endocrinology* 27: 473. 1940.
35. Emery, F. E., and E. L. Schwabe: *Endocrinology* 20: 550. 1936.
36. Emmens, C. W., and A. S. Parkes: *Nature* 143. 1064. 1939.
37. Emmens, C. W., and A. S. Parkes: *J. of Endocrinology* 1: 332. 1939.
38. Euw, J. von, and T. Reichstein: *Helvet chim. acta* 24: 418. 1941.
39. Evans, J. S., R. F. Varney and F. C. Koch: *Endocrinology* 28: 747. 1941.
40. Fagin, J., and S. R. M. Reynolds: *Amer. J. Physiol.* 117: 86. 1936.
41. Fieser, L. F.: The chemistry of natural products related to phenanthrene. Reinhold Publishing Corporation, New York, 1936.
42. Fischer, A.: Rev. franç. d'endocrinol. 16: 1. 1938.
43. Fischer, A., and M. Engel: *Lancet* 236: 354. 1939.
44. Frank, R. T., and H. Sobotka: *Proc. Soc. Exper. Biol. & Med.* 29: 1026. 1932.
45. Frattini, B., and M. Maino: *Biochem. Ztschr.* 253: 202. 1932.
46. Freud, J.: *Biochem. J.* 27: 1438. 1933.
47. Freud, J.: *Acta brev. Neerland.* 8: 130. 1938.
48. Freud, J.: *Acta brev. Neerland.* 9: 11. 1939.
49. Freud, J., S. E. de Jongh and E. Laqueur: Nederl. tijdschr. v. geneesk. 77: 1109. 1933.
50. Gaunt, R.: Am. Soc. Zool. 38th Annual Meet. Philadelphia, Pa., December 1940.
51. Gaunt, R., and H. W. Hays: *Science* 567. 1938.
52. Gaunt, R., W. O. Nelson and E. Loomis: *Proc. Soc. Exper. Biol. & Med.* 39: 319. 1938.
53. Girard, A., G. Sandulesco, A. Fridenson, C. Gaudefroy and I. J. J. Rutgers: *Compt. rend. Acad. d. sc.* 194: 1020. 1932.
54. Girard, A., G. Sandulesco, A. Fridenson and J. J. Rutgers: *Compt. rend. Acad. d. sc.* 195: 981. 1932.
55. Hertz, R., R. K. Meyer and M. A. Spielman: *Endocrinology* 21: 533. 1937.
56. Heuverswyn, J. van, V. J. Collins, W. L. Williams and W. U. Gardner: *Proc. Soc. Exper. Biol. & Med.* 41: 552. 1939.
57. Hirschmann, H., and O. Wintersteiner: *J. Biol. Chem.* 122: 303. 1938.
58. Hoffman, F.: *Zentralbl. f. Gynäk.* 2694. 1938.

59. Hohlweg, W.: *Klin. Wchnschr.* 16: 586. 1937.
60. Hohlweg, W.: *Zentralbl. f. Gynäk.* 63: 1143. 1939.
61. Ingle, D. J.: *Proc. Soc. Exper. Biol. & Med.* 44: 450. 1940.
62. Ingle, D. J.: *Endocrinology* 26: 472. 1940.
63. Inhoffen, H. H., and W. Hohlweg: *Die Naturwiss.* 26: 96. 1938.
64. Inhoffen, H. H., W. Logemann, W. Hohlweg and A. Serini: *Ber. Dtsch. Chem. Ges.* 71: 1024. 1938.
65. Jongh, S. E. de, and L. A. van der Woerd: *Acta brev. Neerland.* 9: 21. 1939.
66. Kathol, J., W. Logemann and A. Serini: *Die Naturwiss.* 25: 682. 1937.
67. Kendall, E. C.: Personal communication.
68. Kendall, E. C.: *Proc. of the Am. Soc. of Biol. Chem.* 33rd Annual Meet. Toronto, Canada, April, 1939.
69. Kendall, E. C.: *Proc. of the Am. Soc. of Biol. Chem.* 34th Annual Meet. New Orleans, Louisiana, March, 1940.
70. Klein, M., and A. S. Parkes: *Proc. Roy. Soc., London, s.B.* 121: 574. 1937.
71. Kofler, A., and A. Hauschild: *Ztschr. f. physiol. Chem.* 224: 150. 1934.
72. Korenchevsky, V., and M. Dennison: *Biochem. J.* 30: 1514. 1936.
73. Korenchevsky, V., M. Dennison and M. Eldridge: *Biochem. J.* 31: 467. 1937.
74. Korenchevsky, V., M. Dennison and K. Hall: *Biochem. J.* 31: 1434. 1937.
75. Korenchevsky, V., and K. Hall: *J. Pathol. & Bact.* 45: 681. 1937.
76. Korenchevsky, V., K. Hall and M. A. Ross: *Biochem. J.* 33: 213. 1939.
77. Kosch, O.: *Arch. f. exper. Pathol. u. Pharmakol.* 77: 432. 1914.
78. Kuizenga, M. H., and G. F. Cartland: *Endocrinology* 24: 526. 1939.
79. Landau, M.: Die Nebennierenrinde. Eine morphologisch-physiologische Studie. Jena, Gustav Fischer, 1915.
80. Lauson, H. D., C. G. Heller, J. B. Golden and E. L. Sevringhaus: *Endocrinology* 24: 35. 1939.
81. Leathem, J. H., and R. C. Crafts: *Endocrinology* 27: 283. 1940.
82. MacCorquodale, D. W., S. A. Thayer and E. A. Doisy: *J. Biol. Chem.* 99: 327. 1933.
83. Marker, R. E.: *J. Am. Chem. Soc.* 60: 1897. 1938.
84. Marker, R. E.: *J. Amer. Chem. Soc.* 62: 3350. 1940.
85. Marker, R. E., O. Kamm, M. Jones and T. S. Oakwood: *J. Am. Chem. Soc.* 59: 614. 1937.
86. Marker, R. E., and E. J. Lawson: *J. Am. Chem. Soc.* 61: 588. 1939.
87. Marker, R. E., and E. Rohrmann: *J. Am. Chem. Soc.* 61: 3592. 1939.
88. Marker, R. E., D. L. Turner, R. B. Wagner, P. R. Ulshafer, H. M. Crooks Jr. and E. L. Wittle: *J. Am. Chem. Soc.* 63: 774. 1941.
89. Marker, R. E., D. L. Turner, R. B. Wagner, P. R. Ulshafer, H. M. Crooks Jr. and E. L. Wittle: *J. Am. Chem. Soc.* 63: 779. 1941.
90. Marrian, G. F.: *Biochem. J.* 23: 1090. 1939.
91. Mason, H. L.: *Endocrinology* 25: 405. 1939.
92. Mason, H. L., W. M. Hoehn, B. F. McKenzie and E. C. Kendall: *J. Biol. Chem.* 120: 719. 1937.
93. Mason, H. L., C. S. Myers and E. C. Kendall: *J. Biol. Chem.* 116: 267. 1936.
94. McPhail, M. K.: *J. Physiol.* 83: 145. 1934.
95. Miescher, K., W. H. Fischer and E. Tschopp: *Biochem. Ztschr.* 300: 14. 1938.
96. Miescher, K., and C. Scholz: *Helvet. chim. acta* 20: 1237. 1937.
97. Miescher, K., and C. Scholz: *Helvet. chim. acta* 20: 263. 1937.
98. Miescher, K., A. Wettstein and E. Tschopp: *Schweiz. med. Wchnschr.* 66: 763. 1936.
99. Morato Manaro, J.: *Arch. de la Clin. e Inst. de Endocrinol. Montevideo* 1: 343. 1940.
100. Mühlbock, O.: Internat. Cong. voor Verlosk. en Gynaecol. Amsterdam, Mei 1938.
101. Müller, G.: Dissertation, Technische Hochschule der Freien Stadt, Danzig, 1938.
102. Mussio Fournier, J. C., A. S. Albrieux and L. Prego: *Arch. de la Clin. e Inst. de Endocrinol.* Montevideo 1: 333. 1940.
103. Mussio, Fournier, J. C., A. S. Albrieux and L. Prego: *Arch. de la Clin. e Inst. de Endocrinol.* Montevideo: 1: 329. 1940.
104. Nathanson, I. T., C. C. Franseen and R. R. Sweeney, Jr.: *Proc. Soc. Exper. Biol. & Med.* 39: 385. 1938.
105. Nelson, W. O., and C. G. Merckel: *Proc. Soc. Exper. Biol. & Med.* 36: 825. 1937.
106. Parkes, A. S.: *Proc. Roy. Soc. London, s.B.* 107: 188. 1930.
107. Parkes, A. S.: *Lancet* 674. 1936.
108. Pearlman, W. H., and O. Wintersteiner: *J. Biol. Chem.* 130: 35. 1939.
109. Pfeiffer, C. A., and C. W. Hooker: *Am. J. Physiol.* 131: 441. 1940.
110. Prins, D. A., and T. Reichstein: *Helvet chim. acta* 24: 396. 1941.
111. Reichstein, T.: *Helvet chim. acta* 19: 1107. 1936.
112. Reichstein, T.: *Helvet chim. acta* 19: 29. 1936.
113. Reichstein, T.: *Helvet chim. acta* 20: 953. 1937.
114. Reichstein, T.: *Helvet. chim. acta* 21: 1490. 1938.
115. Reichstein, T., and J. v. Euw: *Helvet. chim. acta* 21: 1181. 1938.
116. Reichstein, T., and K. Gätzi: *Helvet. chim. acta* 21: 1185. 1938.
117. Robson, J. M.: *J. Physiol.* 92: 371. 1938.
118. Robson, J. M.: *J. Physiol.* 92: 401. 1938.
119. Rubinstein, H. S. A. R. Abarbanel and D. N. Nader: *Proc. Soc. Exper. Biol. & Med.* 39: 20. 1938.
120. Ruzicka, L., M. W. Goldberg, J. Meyer, H. Brüngger and E. Eichenberger: *Helvet. chim. acta* 17: 1395. 1934.
121. Ruzicka, L., M. W. Goldberg and H. R. Rosenberg: *Helvet. chim. acta* 18: 1487. 1935.
122. Ruzicka, L., and K. Hofmann: *Helvet. chim. acta* 20: 1280. 1937.
123. Ruzicka, L., K. Hofmann and H. F. Meldahl: *Helvet. chim. acta* 21: 597. 1938.

124. Ruzicka, L., K. Hofmann and H. F. Meldahl: *Helvet. chim. acta* 21: 371. 1938.
125. Ruzicka, L., and H. Kägi: *Helvet. chim. acta* 19: 842. 1936.
126. Ruzicka, L., and H. Kägi: *Helvet. chim. acta* 20: 1557. 1937.
127. Ruzicka, L., M. Oberlin, H. Wirz and J. Meyer: *Helvet. Chim. acta* 20: 1283. 1937.
128. Ruzicka, L., and H. R. Rosenberg: *Helvet. chim. acta* 19: 357. 1936.
129. Ruzicka, L., and A. Wettstein: *Helvet. chim. acta* 18: 986. 1935.
130. Ruzicka, L., and A. Wettstein: *Helvet. chim. acta* 18: 1264. 1935.
131. Ruzicka, L., and A. Wettstein: *Helvet. chim. acta* 19: 1141. 1936.
132. Salmon, U. J., and H. Geist: *Proc. Soc. Exper. Biol. & Med.* 45: 522. 1940.
133. Schacher, J., J. S. L. Browne and H. Selye: *Proc. Soc. Exper. Biol. & Med.* 36: 488. 1937.
134. Selye, H.: Unpublished.
134b. Selye, H.: Am. Assoc. Anat. 56th Annual Meet., Louisville, Kentucky, March, 1940.
135. Selye, H.: *Science* 94: 94. 1941.
136. Selye, H.: *Proc. Soc. Exper. Biol. & Med.* 46: 142. 1941.
137. Selye, H.: *Proc. Soc. Exper. Biol. & Med.* 46: 116. 1941.
138. Selye, H.: *J. Pharmacol. & Exper. Therap.* 71: 236. 1941.
139. Selye, H.: *Nature* 148: 84. 1941.
140. Selye, H.: *J. Urol.* 46: 110. 1941.
140b. Selye, H., and S. Albert: Canad. Physiol. Soc. Meeting, October, 1941.
141. Selye, H., and S. Friedman: *Am. J. Cancer* 38: 558. 1940.
142. Selye, H., and S. Friedman: *Endocrinology* 28: 129. 1941.
143. Selye, H., C. M. Harlow and J. B. Collip: *Endokrinologie* 18: 81. 1936.
144. Selye, H., and G. Masson: *Endocrinology* 25: 211. 1939.
144b. Selye, H., and V. Schenker: *Proc. Soc. Exper. Biol. & Med.* 39: 518. 1938.
145. Serini, A., and W. Logemann: *Ber. Dtsch. Chem. Ges.* 71: 186. 1938.
146. Slotta, K. H., H. Ruschig and E. Fels: *Helvet. chim. acta* 17: 1361. 1934.
147. Soderwall, A. L., R. J. Blandau and E. S. Jordan: *Am. Soc. Zool.* 38th Annual Meet. Philadelphia, Pa., December, 1940.
148. Spurr, C. L., and C. D. Kochakian: Proc. of the Am. Physiol. Soc. 50th Annual Meet. Baltimore, Md., March, 1938.
149. Spurr, C. L., and C. D. Kochakian: *Endocrinology* 25: 782. 1939.
150. Stavely, H. E.: *J. Amer. Chem. Soc.* 61: 79. 1939.
151. Steiger, M., and T. Reichstein: *Helvet. chim. acta* 20: 1164. 1937.
152. Steiger, M., and T. Reichstein: *Helevt. chim. acta.* 21: 546. 1938.
153. Stewart, G. N.: Reported by MacCallum, Inter. Med. Congress, Div. III, London, 1913.
154. Stolk, D. van, and H. Pénau: *J. Pharmacie* 24: 155. 1936.
155. Thayer, S. A., D. W. MacCorquodale and E. A. Doisy: *J. Pharm. & Exper. Therap.* 59. 48. 1937.
156. Tschopp, E.: *Nature* 136: 258. 1935.
157. Tschopp, E.: *Arch. Internat. de pharmacodyn. et de thérap.* 52: 381. 1936.
158. Venning, E. Hill: *J. Biol. Chem.* 119: 473. 1937.
158b. Waelsch, H., and H. Selye: *Naunyn-Schmiedebergs Arch.* 161: 115. 1931.
159. Waterman, L., M. Danby, J. H. Gaarenstroom, R. W. Spanhoff and I. E. Uyldert: *Acta brev. Neerland.* 9: 75. 1939.
160. Wells, J. A., and R. R. Greene: *Endocrinology* 25: 183. 1939.
161. Wintersteiner, O., and J. J. Pfiffner: *J. Biol. Chem.* 116: 291. 1936.
162. Wolfe, J. K., L. F. Fieser and H. B. Friedgood: *J. Amer. Chem. Soc.* 63: 582. 1941.
163. Yanagita, T.: *Tokyo Igakkai Zasshi* 51: 901. 1937.
164. Zéphiroff, P., N. Dobrovolskaïa-Zavadskaïa and C. Drosdovsky: *Compt. rend. Soc. de biol.* 132: 378. 1939.
165. Zondek, B.: Die Hormone des Ovariums und des Hypophysenvorderlappens. Springer, Berlin, 1931.

INFLUENCE OF TESTOSTERONE ON THE MORPHOGENETIC ACTIONS OF ESTRADIOL

S. ALBERT

From the Department of Anatomy, McGill University

MONTREAL, QUEBEC, CANADA

IT IS WELL KNOWN that while some of the morphological actions of folliculoids[1] are inhibited, others are augmented by testoids.[1] In an effort to undertake a systematic study of these interrelations, the organs of rats receiving estradiol, either alone or in combination with testosterone, have been examined. A preliminary study of these interrelations in postpubertal rats was made by Selye (1). It was felt, however, that the morphogenetic effects of these hormones may become even more evident if treatment is started before puberty and continued until sexual maturity is attained. The present communication reports on experiments performed under these conditions.

EXPERIMENTAL PROCEDURE

Twenty-four prepubertal male albino rats weighing 65 to 90 gm. (average 78 gm.) were divided into 4 equal groups as follows. *Group I* was killed before injections were started and served as pre-experimental controls showing the condition of the organs at the onset of treatment. *Group II* was likewise not treated and served as post-experimental controls indicating the normal development of the organs during the experimental period. *Group III* was treated with two daily subcutaneous injections of 150 micrograms of α-estradiol dissolved in 0.1 cc. of peanut oil. *Group IV* received the same amount of estradiol as the preceding group and in addition to this, two daily subcutaneous injections of 5 mg. of testosterone in 0.1 cc. of peanut oil. After 24 days of this treatment *Groups II, III* and *IV* were killed with chloroform. The pituitary, adrenals, thymus, testes, seminal vesicles, prostate, epididymis, preputial glands, coagulating glands and kidney were dissected and weighed after 24 hours of fixation in 'Susa' mixture. Sections of these organs were stained with the hematoxylin-eosin method after embedding in paraffin.

The average body weight and the average weight of all these organs are recorded in table 1 together with the spread which is indicated in brackets.

The figures in table 1 indicate that estradiol caused a marked inhibition of body growth which was only partly prevented by simultaneous testosterone treatment. There was also some inhibition by testosterone of the increase in the pituitary and adrenal weight caused by estradiol. The thymus, on the other hand, underwent much more pronounced involution in animals receiving estradiol in combination with testosterone than in those treated only with the former. The testes of the estradiol-treated animals showed no growth during the entire experimental period; indeed, their average weight after 24 days of treatment with the folliculoid hormone was ac-

Received for publication January 6, 1942.

[1] The terms 'folliculoid' instead of 'female hormone-like' or 'estrogenic' and 'testoid' instead of 'male hormone-like' or 'androgenic' are used here in agreement with the recently proposed pharmacological classification of the steroid hormones (2).

TABLE 1. SUMMARY OF ORGAN WEIGHT CHANGES FOLLOWING TREATMENT WITH ESTRADIOL AND TESTOSTERONE

	6 Pre-experimental controls	6 Post-experimental controls	6 Estradiol-treated	6 Estradiol and testosterone-treated
Initial body wt., *gm.*	78 (75–80)	78 (65–90)	78 (65–90)	79 (70–85)
Final body wt., *gm.*		168 (140–205)	121 (105–140)	144 (135–155)
Pituitary wt., *mg.*	3 (2–3)	6 (4–7)	8 (7–9)	6 (4–8)
Adrenal wt., *mg.*	24 (21–28)	34 (30–37)	42 (37–49)	36 (24–47)
Thymus wt., *mg.*	205 (190–258)	382 (268–476)	110 (76–130)	56 (33–70)
Testis wt., *gm.*	328 (218–464)	2.065 (1.676–2.294)	276 (178–543)	1.190 (987–1.578)
Seminal vesicles wt., *mg.*	7 (5–9)	214 (112–335)	48 (43–56)	787 (702–908)
Anterior prostate wt., *mg.*	19 (8–23)	135 (75–206)	10 (8–13)	361 (280–444)
Posterior prostate wt., *mg.*[1]	27 (19–26)	150 (85–213)	37 (25–60)	429 (357–488)
Epididymis wt., *mg.*	53 (42–61)	180 (225–337)	68 (55–79)	354 (282–390)
Preputial glands wt., *mg.*	24 (14–31)	65 (45–90)	37 (26–48)	90 (72–108)
Coagulating glands wt., *mg.*	3 (2–4)	46 (44–67)	14 (10–20)	162 (142–179)
Kidney wt., *gm.*	929 (802–966)	1.745 (1.392–2.142)	1.383 (1.178–1.633)	1.984 (1.642–2.181)

[1] Including coagulating gland

TABLE 2. HISTOLOGICAL SUMMARY

Group, Treatment	Testis		Seminal vesicles	Anterior prostate	Posterior prostate	Kidney
	Interstitial cells	Tubules				
Pre-experimental, controls	Small	Diameter, narrow. No sperm or spermatids	Muscular fibres thin; lumen, narrow; no secretion; epith. cells poor in cytoplasm	Stroma poorly developed; acini narrow; some secretion; moderate amount of cytoplasm in epith. cells	Stroma poorly developed; acini small with scant secretion; moderate amount of cytoplasm in epith. cells	Normal for immature rat
Post-experimental, controls	Normal	Diameter, wide. Complete spermatogenesis	Muscular fibres thin; lumen wide; copious secretion; epith. cells rich in cytoplasm; secretion granules	Stroma poorly developed; acinar lumen distended with secretion; epith. cells rich in cytoplasm	Stroma very poorly developed; acini distended with secretion; epith. cells rich in cytoplasm	Normal for adult rat
Estradiol	Small	Diameter, narrow. No sperm or spermatids	Muscular fibres thick; lumen narrow; no secretion; epith. cells poor in cytoplasm	Stroma poorly developed; acini narrow; slight secretion; epith. cells poor in cytoplasm	Stroma very well developed; acini small; fair secretion; epith. cells contain fair amount of cytoplasm; lymphocytic infiltration	Cortex relatively small; tubular epith. low
Estradiol and testosterone	Atrophic	Diameter, wide. Complete spermatogenesis	Muscular fibres thin; lumen distended with enormous amount of secretion; epith. cells rich in cytoplasm; secretion granules	Stroma poorly developed; acini very distended; epith. cells rich in cytoplasm	Stroma well developed; acini distended; epith. cells rich in cytoplasm	Cortex extremely well developed, larger than in controls; tubular epith. high

tually smaller than that of prepubertal control animals killed at the beginning of the experiment. However, simultaneous treatment with testosterone not only prevented

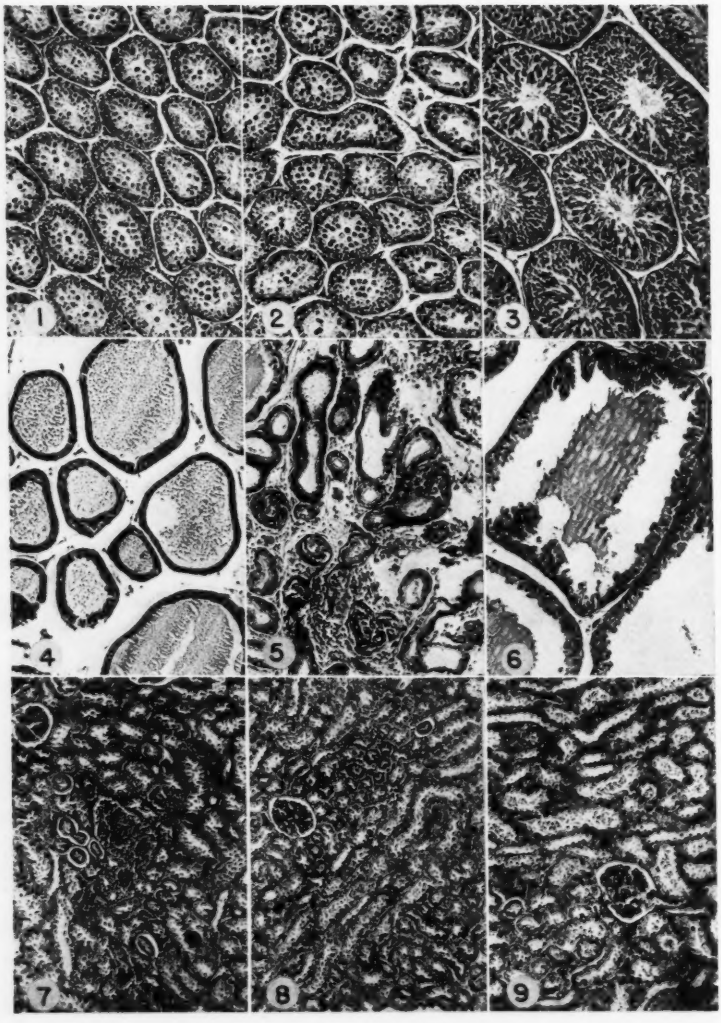


Fig. 1–3. PHOTOMICROGRAPHS OF RAT TESTIS. Fig. 1. CONTROL RAT at the beginning of the experiment. Fig. 2. ESTRADIOL-TREATED RAT. Lack of development of the seminal epithelium. Fig. 3. RAT RECEIVED TESTOSTERONE IN ADDITION TO ESTRADIOL. Seminal epithelium fully developed.

PHOTOMICROGRAPHS OF POSTERIOR PROSTATE GLAND. Fig. 4. CONTROL RAT at the conclusion of the experiment. Fig. 5. ESTRADIOL-TREATED RAT showing stroma proliferation, atrophic epithelium and areas of lymphocytic infiltration in the stroma. Fig. 6. RAT TREATED WITH TESTOSTERONE in addition to estradiol. Stroma normal, epithelium fully developed and secreting.

PHOTOMICROGRAPHS OF KIDNEY. Fig. 7. NORMAL CONTROL RAT at the conclusion of the experiment. Fig. 8. ESTRADIOL-TREATED RAT, narrow convoluted tubules, lined by low epithelium. Fig. 9. RECEIVED TESTOSTERONE IN ADDITION TO ESTRADIOL. Tubules dilated, epithelium high. Typical renotropic action.

this involution, but permitted a marked increase in testis weight although this was not quite as pronounced as in the untreated controls. The seminal vesicles, posterior

prostate, epididymis, preputial glands and coagulating glands all showed some increase in weight in spite of estradiol treatment, while the anterior prostates were actually smaller after treatment than at the onset of the experiment. All male accessory sex organs were considerably larger than normal in the group receiving estradiol in combination with testosterone. The growth of the kidneys was not completely prevented, but greatly inhibited, by estradiol treatment but the renotropic action of testosterone was not prevented by the simultaneous administration of estradiol so that the average kidney weight in the group receiving both types of hormones was actually greater than that of untreated controls of the same age.

The most important results of the histological study of the testis, seminal vesicles, prostate and kidney are summarized in table 2.

It appears that while estradiol completely prevented the maturation of the testis, simultaneous treatment with testosterone permitted the development of a normal spermatogenic epithelium. This indicates that testosterone given simultaneously with estradiol not only prevents the destruction of an already formed spermatogenic epithelium in the adult animal (1) but even permits the maturation of such an epithelium at puberty (fig. 1, 2, 3).

The increase in the weight of the accessory sex organs after estradiol treatment proved to be due to proliferation of the connective tissue and smooth muscle elements in the stroma. This change, previously described by Lacassagne (3), Burrows (4) and de Jongh (5), was marked in all accessory sex organs except the anterior prostate which consequently showed no increase in weight. In most cases marked round cell infiltration of the stroma of the posterior prostate was evident in this group and the epithelium in the vicinity of the urethra showed squamous metaplasia. The animals receiving testosterone in addition to estradiol showed no stroma proliferation or lymphocytic infiltration, but marked stimulation of the secretory epithelium (fig. 4, 5, 6). Contrary to the findings of Bühler (6) who claimed that the prostatic metaplasia caused by estrone is prevented by testosterone, in our experiments the metaplasia was not prevented.

The kidneys of the estradiol-treated animals were characterized by a particularly narrow cortex with very low cells in the proximal and distal convoluted tubules. The medulla of the kidney showed no significant change. Simultaneous treatment with testosterone revealed the usual renotropic effect (7).

SUMMARY

In male rats receiving estradiol either alone or in combination with testosterone beginning before the onset of puberty the following observations were made.

The enlargement of the pituitary and adrenals caused by estradiol is inhibited by simultaneous testosterone administration.

The thymus atrophy caused by estradiol is intensified in the case of concurrent treatment with testosterone.

The pubertal development of the testes is inhibited by estradiol but in spite of such treatment complete spermatogenesis occurs at puberty in animals receiving testosterone simultaneously with estradiol. Treatment with this testoid has no influence, however, on the interstitial cell atrophy caused by the folliculoid compound.

The marked proliferation of the stroma elicited by estradiol in the male accessory sex organs is confirmed and it is pointed out that pronounced round cell infiltration in the posterior prostate may be caused by the hormone. All of these changes are prevented by simultaneous testosterone treatment. The squamous metaplasia of the prostatic epithelium which is caused by folliculoid substances was not prevented by testosterone, a fact which is in contradiction to previous reports on this subject.

The renotropic action of testosterone is not prevented by simultaneous estradiol treatment.

The expenses of this investigation were defrayed by the Blanche E. Hutchinson Fund of McGill University administered by Professor Hans Selye. I am greatly indebted to Drs. Gregory Stragnell and Erwin Schwenk of the Schering Corporation of Bloomfield, New Jersey, who supplied the estradiol and testosterone used in this work.

REFERENCES

1. Selye, H.: *Canad. M. A. J.* 42: 113. 1940.
2. Selye, H.: *Nature* 148: 84. 1941.
3. Lacassagne, A.: *Compt. rend. Soc. de Biol.* 113: 590. 1933.
4. Burrows, H.: *J. Path. & Bact.* 41: 423. 1935.
5. de Jongh, S. E.: *Acta brev. Neerland.* 3: 112. 1933.
6. Bühler, F.: *Ztschr. f. d. ges. exper. Med.* 104: 249. 1938.
7. Selye, H.: *J. Urol.* 42: 637. 1939.

UTERINE AND OVARIAN RESPONSE TO PARTIAL AND SUBTOTAL OVARIAN RESECTION[1]

G. VAN WAGENEN AND ARTHUR H. MORSE
From the Department of Obstetrics and Gynecology, Yale University School of Medicine
NEW HAVEN, CONNECTICUT

DURING THE PAST FEW YEARS clinical and experimental papers have been published respecting the effect upon the reproductive processes of the partial resection of ovarian tissue. Among clinicians there is a lack of unanimity of opinion regarding the effect of this procedure. Hendry (1), for example, states that the amount of tissue necessary for the conservation of menstrual function, as for fertility, is very small. He adds that, after a unilateral oöphorectomy with the uterus conserved there is rarely a gap in the menstrual cycle, and the small residue of a single remaining ovary tends to hypertrophy and maintains menstrual function for an indefinite period.

However, Runge (2) and Caffier (3) have expressed a different viewpoint. Runge believes that both ovaries are essential for the preservation of a normal genital function. He regards the presence of cysts in the remaining ovary as a typical change following a unilateral ovariectomy; and believes that the disturbed processes of follicle ripening, and of corpus luteum formation and degeneration, which now no longer alternate between two glands, bear a causative relation to cyst formation in the ovary. Caffier holds that a single ovary cannot always assume the function of both; and that it is important whenever possible to conserve both ovaries. In more than two thirds of a series of 174 women, this author records an alteration of menstruation following unilateral oöphorectomy, and lessened fertility as the age of the patient increased.

Experimentally similar pathological findings have been reported. Lipschütz (4) found in the cat and guinea pig that, following the removal of one ovary and the subtotal resection of the other, the growing follicles became cystic while the surrounding tissue underwent luteinization. The changes were sometimes associated with intrafollicular hemorrhage. More striking, however, was the histological character of the uterine endometrium which resembled 'grandular cystic hyperplasia of the human.' Burch (5) and his coworkers observed in one group of partially castrated guinea pigs an atrophic endometrium tending toward the castrate type and, in a second group, a glandular cystic hyperplasia comparable to that seen in the human uterus. Hartman (6) reports that the reduction of the ovarian mass beyond semicastration is not compatible with normality of function, and adds that the relative excess of anterior pituitary hormone injures the follicle which readily becomes cystic.

The majority of experimental investigators of this problem have employed the smaller laboratory animals which have estrous cycles, and which, because of the presence of multiple fetuses, have a greater amount of uterine endometrium and musculature to be supported. Our observations have been made on the macaque monkey. The genital tract of this animal anatomically and functionally resembles that of the human.

Received for publication December 22, 1941.
[1] This work was aided by a grant from the Research Fund of the Yale University School of Medicine.

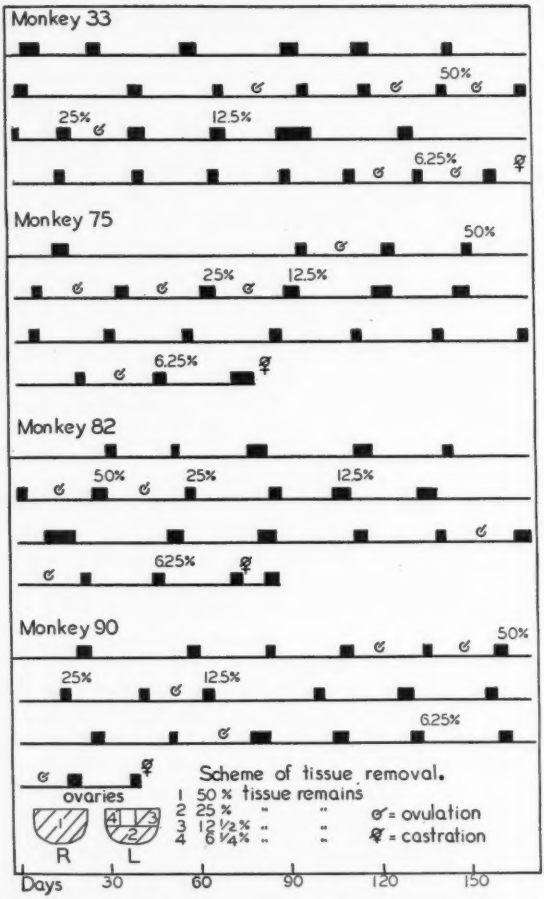


Fig. 1. THE CHART DEPICTS THE MENSTRUAL HISTORIES (solid areas represent vaginal bleeding) of 4 animals used in this experiment. Ovulation estimated by a follicle rupture point and the presence of a corpus luteum at laparotomy is shown by a symbol. When ovarian tissue was excised, the percentage recorded indicates the amount of tissue which remained to support reproductive activity. The scheme of removal was first to excise one ovary, then to reduce the second according to the successive figures as shown in the diagram of the two ovaries (see key to chart). In all monkeys the ovarian remnant was removed at operation, but in only one animal (*monkey 82*) did this castration precipitate an involutional bleeding on the fifth day.

There is a menstrual cycle of similar length, only one ovulation takes place in a cycle and, of course, only one offspring is produced at a time.

EXPERIMENTAL PROCEDURE

In the present study, the question of primary interest concerned the percentage of total ovarian tissue which could be removed without interfering with menstruation and ovulation. It was planned to study the character of the menstrual cycles and at the same time to observe directly by laparotomy the remaining ovary or its remnant. Thus it could be told to what length it was possible to proceed in the reduction of

tissue before the physiology of the gland and the reproductive activity of the animal was impaired. During the exploratory operations attention was also directed to the occurrence of compensatory hypertrophy and of cystic changes in the ovary. The occurrence of ovulation, as evidenced by the presence of a corpus luteum formed since a preceding observation, was the criterion indicating full functioning of the ovary.

Four adult rhesus monkeys having menstrual cycles normal in character were chosen for this investigation. Further preliminary exploratory laparotomy made certain that these animals were ovulating, and from three to eight cycles were observed before the monkeys were unilaterally ovariectomized. In each case the ovary containing the most recent corpus luteum was left in situ. Therefore, the weight of the excised ovary was the smaller and, without the increment of a developing follicle or

TABLE 1. COMPARISON OF WEIGHTS OF THE TWO OVARIES OF EACH EXPERIMENTAL ANIMAL. The first ovary was removed as a whole and the second in four parts. In later operations it was impossible to avoid inclusion of luteal tissue

	Monkey 33		*Monkey 75*		*Monkey 82*		*Monkey 90*	
Ovaries	Left	Right	Right	Left	Right	Left	Right	Left
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Parts								
1		108.8		112.6		108.8		080.6
2		026.4		088.1		039.4		098.2[1]
3		239.4[1]		144.6[1]		134.8[1]		063.0
4		428.9[1]		307.8[1]				
Total	164.2	803.5	187.2	643.1	137.2	283.0	168.5	241.8

[1] Includes corpus luteum tissue.

corpus luteum, was more nearly the basic weight of the gland. Since an earlier examination in each animal revealed no real difference in size between the ovaries, it was believed fair to estimate that by unilateral ovariectomy 50 per cent of the ovarian tissue for that particular animal had been removed. In no case did unilateral ovariectomy precipitate uterine bleeding, as it sometimes does, nor was it followed by irregular cycles. In one case ovulation was known to have taken place a few days after operation and within the operative cycle.

When continuance of regular menstrual cycles was assured and ovulation had been confirmed, the quantity of ovarian tissue was again reduced by half, 25 per cent of the original tissue then remaining. The resting ovary of the rhesus monkey is flattened and somewhat the shape of a Lima bean. In this second operation (see scheme of tissue removal in fig. 1) the half of the gland which lies away from the ovarian blood vessels and nerve supply was removed. All operations were performed early in the cycle before follicular development had begun, allowing time for the maximum degeneration of the corpus luteum of the preceding cycle. In the repeated operations upon the second ovary corpora were necessarily present, but by directing the cut to one side or the other it usually was possible to avoid inclusion of luteal tissue.

At the third operation it was necessary to approach the tissue of the hilus and the position of the degenerating corpus luteum determined which pole of the ovary was to remain. When the third excision was completed, one ovary and three-fourths of the second gland had been removed, so that only 12½ per cent of the original quantity of ovarian tissue remained. These first three excisions followed in quick succession during the midwinter months, when, as was found by Hartman, menstrual cycles are comparatively regular and the rhesus is most likely to be ovulating frequently. Since

amenorrhea and prolonged cycles during the summer months have been reported frequently for the rhesus monkey, it was thought advisable to let the animals rest a year to ensure adequate evaluation of the menstrual cycles as to length and quantitative bleeding.

In figure 1 the third line of the menstrual record of each animal covers the summer months following surgical interference. *Monkey* 33 had two extended cycles of 42 and 55 days during May, June and July respectively. Likewise *monkey* 82 ran cycles of 44 and 41 days during April, May and June and *monkey* 92 ran a single long cycle of 39

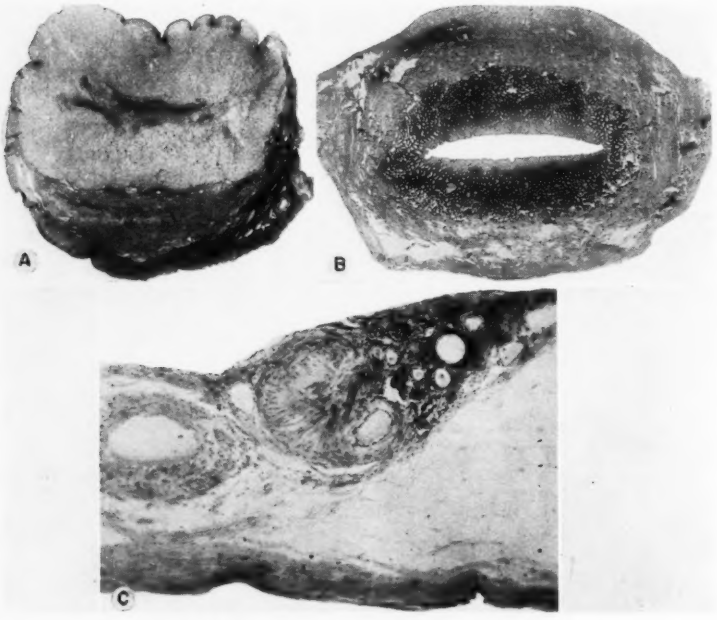


Fig. 2. A. Ovarian remnant showing corpus luteum, from *monkey* 33.
B. Uterus from *monkey* 33 to show maintenance of size of uterus and depth of endometrium.
C. Accessory ovarian tissue found in *monkey* 90.

days during June. There was no protracted amenorrhea in any one of these four animals during the summer months. Even *monkey* 75 showed no long cycle, although during the previous summer this animal, after arrival from our animal dealer, had had no bleeding throughout a period of 82 days. Therefore, during eight months, cycles which were normal in character, as judged by their length and by the amount and duration of menstrual bleeding, depended upon only 12½ per cent of the original quantity of ovarian tissue. Rectal palpation at the end of this time indicated uteri of normal size.

It was then determined to perform laparotomies and, if corpora lutea were found, to excise one-half of the already reduced ovary. In each animal one or more corpora lutea were found. In the case of *monkeys* 33 and 75 the most recent stigma was large and well vascularized, and the corpus luteum was of such size that we felt it could be safely assigned to the preceding cycle. In *monkey* 82 the corpora were older but cer-

tainly formed in the last few cycles during the three-quarters of the year which had elapsed since the third operation. Our greatest difficulty in proceeding with the plan of reduction by half of the remaining ovarian tissue was encountered in animals 82 and 90 in which the ovarian tissue was involved in adhesions. The fourth excision was not uniform, but, as far as possible, the tissue removed was stroma as well as luteal, and the incision lay sufficiently distant from the hilar blood vessels to insure continued vascular supply to that part of the ovary which remained.

Hypertrophy of the remaining ovarian tissue was plainly recognizable only after

TABLE 2. MEASUREMENTS OF THE OVARIES AT THE BEGINNING OF THE EXPERIMENT IN RELATION TO THE ANIMAL'S WEIGHT AND THE SIZE OF THE UTERUS, INDICATING THE INCREASE IN BODY WEIGHT AND THE MAINTENANCE OF UTERINE SIZE, IN SPITE OF REPEATED LAPAROTOMY AND THE RADICAL RESECTION OF OVARIAN TISSUE.

	Beginning of Experiment	End of Experiment
M33	Body weight 4400 gm. Ovaries *R*—0.8×0.6×0.4 cm. (corpus luteum) *L*— — — — — — — Uterus—1.8=1.0=2.2 cm.	5650 gm. (gain=1250 gm.) 1.95×1.3×3.4 cm.
M75	Body weight 5170 gm. Ovaries *R*—0.9×1.1×.25 cm. *L*—1.1×0.7×0.5 cm. (foll.) Uterus—1.6×1.1×2.5 cm.	6970 gm. (gain=1800 gm.) 2.1×1.3×2.6 cm.
M82	Body weight 3990 gm. Ovaries *R*—0.8×0.4×0.4 cm. *L*—1.0×0.6×0.5 cm. (corpus luteum) Uterus—1.8×1.3×2.6 cm.	5100 gm. (gain=1110 gm.) 1.8×1.3×2.5 cm.
M90	Body weight 5255 gm. Ovaries *L*—1.2×0.9×0.8 cm. (corpus luteum) *R*—0.95×0.5×0.4 cm. Uterus—1.5×1.1×2.1 cm.	6900 gm. (gain=1645 gm.) 1.6×1.1×2.2 cm.

the 8-month period of rest. Theoretically after rapid reduction of the second ovary only one-quarter of that ovary remained. However, on opening these animals 8 months later the ovarian fragment was found to be as large, or larger, than the normal ovary. Adhesions obscured the outlines of two ovaries and in the other two cases the gland appeared discrete but irregular in shape. The hypertrophy possibly was due to the production of the usual number of growing follicles of each cycle in the small remaining portion of stroma and to the presence of new and regressing corpora. It seems probable that corpora do not regress in size so rapidly after surgical procedures upon the ovary.

It would have been entirely unsatisfactory to carry on further excisions because of the two cases cited in which the ovarian tissue was not discrete. Only one new cycle for each animal was recorded, and then the ovarian remnant was removed. The cycles following the last operative procedure were remarkably regular, 23, 28, 26 and 27 days.

At the end of the experiment the ovarian remnant was removed together with the fundus of the uterus. In only one case (*monkey 82*) was there an involutional bleeding, which occurred on *day 12* of the concurrent cycle and five days after castration. The bleeding continued for five days.

Histological examination of the ovarian tissue removed at final operation indicated that ovulation had taken place in *monkeys* 33 and 90. The stroma which had remained after the earlier excision was scanty. At this time the first evidence of cystic degeneration was found in *monkeys* 82 and 90.

The measurements of the uteri removed at the end of the experiment are compared with measurements made at the beginning of the experiment in table 2. It will be seen that the size had been maintained. The endometrium was normal in depth. The only comments to be made were that in *monkey* 33 there were more glands than are usually seen at any time in the normal cycle. This uterus had been removed on the 19th day, which was later in the cycle than the laparotomies were usually performed. Many of the glands were beginning to be sacculated, some were overdistended. The lumen of the uterus of *monkey* 82 was dilated, causing a lessened depth of epithelium. The stroma was edematous, the glandular epithelium was proliferating rapidly and the deeper glands were dilated. Dilated basal glands were found also in the uterus of *monkey* 75.

The rhesus monkey tolerates surgical intervention remarkably well. The body weight of these animals, as given in table 2, was taken at the time of the semicastration. From that time until the end of the experiment 14 months elapsed, so that the gains of 1250, 1800, 1110 and 1645 grams (average 1451 grams) represent the progress of animals undergoing four or five laparotomies during little over a year's time. The average gain of 10 control animals living in the same colony was little more than this.

Corner has described delicate adhesions extending from the visceral peritoneum to the extruded part of a corpus luteum. In the series of laparotomies performed in this study, threadlike adhesions to stigmata were found in two instances. In the first case, *monkey* 75, no tissue had been removed; in the second, *monkey* 82, only the ovary on the opposite side had been removed. The adherence of the stigma of a corpus luteum to the contiguous peritoneum results in the formation of adhesions, which become attenuated because of the movements of the viscera, and such adhesions may occasionally be seen by anyone who makes numerous observations on monkey ovaries.

SUMMARY

Twelve and one-half per cent of the total amount of ovarian tissue sustained menstrual cycles normal in length and character of bleeding in rhesus monkeys. Frequent ovulation took place in the ovarian fragments and palpation indicated the uterus in each animal to be normal in size. Further reduction of ovarian tissue to 6¼ per cent was followed by regular cycles and ovulation in two instances. The endometrium of each uterus was essentially normal for the particular phase of the cycle during which it was removed.

Hypertrophy, as suggested by comparison of the sum of the weights of the parts of the second ovary to the whole of the ovary, appeared on histological examination to be largely due to the necessary inclusion of the degenerating follicles and corpora lutea.

In one animal accessory ovarian tissue was found in the broad ligament.

REFERENCES

1. Hendry, J.: *J. Obst. & Gynec. Brit. Emp. New Series* 43: 609. 1936.
2. Runge, H.: *Monatschr. f. Geburtsh. u. Gynäk.* 100: 233. 1935.
3. Caffier, P.: *Zentralbl. f. Gynäk.* 61: 2370. 1937.
4. Lipschütz, A.: *Gynéc. et obst.* 36: 408. 1937.
5. Burch, J. C., G. S. McClellan, C. D. Johnson and E. T. Ellison: *J. Am. Med. Assoc.* 108: 96. 1937.
6. Hartman, C. G.: *J. Mammalogy* 12: 129. 1931.

SEX HORMONES AND HAIR CHANGES IN RATS

THOMAS R. FORBES

From the Department of Anatomy, Johns Hopkins University

BALTIMORE, MARYLAND

THE PROLONGED INJECTION of estradiol benzoate into male black rabbits has been reported to cause, after two and one-half to five months, first a change in hair color from black to red and then the development of symmetrical areas of alopecia; such effects were not seen in similarly treated female rabbits (1). There appears to be no information in the literature regarding hair changes in rats treated with estrogens and androgens. The observations to be reported, made incidental to experiments involving the administration of sex hormone pellets, seem worthy of record.

MATERIALS AND METHODS

The results to be described were observed during the prolonged treatment of normal adult male and female rats (2) with subcutaneous pellets of the following crystalline substances: testosterone dipropionate, androsterone, estrone, α-estradiol, α-estradiol benzoate, α-estradiol dipropionate, estriol, equilin, diethylstilbestrol methyl ether (α,α'-diethyl-4-hydroxy-4'-methoxy-stilbene), diethylstilbestrol methyl ether acetate (α,α'-diethyl-4-acetyl-4'-methoxy-stilbene), and diethylstilbestrol dipropionate.[1] The pellets, in most cases weighing 8 to 10 mg., were prepared by a uniform procedure, for full technical details of which reference should be made to the original report (2). Each pellet was carefully weighed before implantation and after recovery at autopsy in order to determine the amount of hormone absorbed. Only one pellet was implanted in each animal.

The rats were either albino or of a strain, developed by crossing wild and laboratory rats, with dark grey-brown hair over all the body except on the abdomen, where the hair was usually white.[2]

RESULTS

The results to be described were not observed in large groups of control rats maintained with the experimental animals under identical conditions.

Pellets were not always removed as soon as hair changes were detected, i.e., the periods of treatment reported below were sometimes longer than was necessary for the production of the observed hair changes. However, the minimum periods of treatment indicated for each compound approximate in most cases the time required, under the conditions of the experiment, to produce the minimal results described.

Received for publication December 31, 1941.

[1] The author is most grateful to the following for supplying the hormones used: Dr. Erwin Schwenk of the Schering Corp., Bloomfield, N. J., for androsterone, α-estradiol, α-estradiol benzoate, estrone and estriol; Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N. J., for androsterone, α-estradiol dipropionate, testosterone dipropionate and equilin; Dr. John M. Shaul of the Winthrop Chemical Co., Inc., New York City, for diethylstilbestrol dipropionate; Dr. Franz C. Schmelkes, Wallace & Tiernan Products, Inc., Belleville, N. J., for diethylstilbestrol monomethyl ether and diethylstilbestrol monomethyl ether monoacetate.

[2] Acknowledgment is made to the Department of Biology of Swarthmore College for generously providing animals, quarters, and facilities for carrying out much of this work.

Testosterone dipropionate. No hair changes were detected in rats of the dark grey-brown strain that had absorbed more than 7 mg. over periods exceeding five months.

Androsterone. In two male and two female albino rats that had absorbed 6.4 to 8.4 mg. of the hormone in 94 to 127 days, the previously white hairs in many areas of the body had become either a diffuse light red-brown or, particularly on the scruff of the neck, a dark red-brown on the distal third and white on the basal two-thirds. The latter type of response was interpreted as indicating only transitory pigment deposition. There was no constant pattern for the sites of the pigmented areas, although the latter were symmetrical on a given animal. In addition, one of the above females, and another female that had absorbed 3.9 mg. of androsterone in 47 days, showed a striking concentration of black pigment in the capsule characteristically forming around the pellet (2) and in the immediately adjacent connective tissue.

Estrone. Of four albino rats which carried pellets for more than 150 days, one male (151 days, absorbed 5.0 mg.) had moderate tan, red, and brown coloration of many of the body hairs and large areas of alopecia in which were empty, brown-pigmented hair follicles. A female (258 days, 7.3 mg.) had moderate brownish coloration of many hairs but no hair loss. Neither this animal nor other alopecic rats to be described showed any gross signs of skin disease. An additional male (350 days, 4.0 mg.) and female (400 days, 3.2 mg.) showed no hair changes, but the capsule of the pellet in the male had a uniform light brown color throughout.

α-Estradiol. Two male albino rats (152 days, 4.6 mg.; 180 days, 6.5 mg.) had in one case moderate, and in the other case marked, tan, red-brown, and brown coloration of about half their fur, as well as some hairs tipped with dark red-brown. Three males (182–315 days, 3.0–4.2 mg.) of the dark grey-brown strain showed extensive, nearly symmetrical areas of alopecia.

α-Estradiol benzoate. Of three male albinos (168, 186, 367 days; 4.3, 4.6, 7.6 mg., respectively), two (168, 186 days) had light brown hair over the greater part of their bodies, and all three had hairs tipped with brown on their necks. In addition, one of the males (186 days) had a pellet capsule that was an intense black over half its surface, while the mammary glands, bilaterally well developed, were a dark grey to black throughout so that the ducts and alveoli were clearly delineated. Three males of the dark grey-brown strain (182–355 days, 2.2–3.0 mg.) showed conspicuous, very extensive alopecic areas.

α-Estradiol dipropionate. Two male and two female albino rats (108–185 days, 2.9–7.6 mg.) had scanty to moderate numbers of grey-tan and brown hairs over parts or all of their bodies.

Estriol. In two albino females (73 days, 2.9 mg.; 93 days, about 7 mg.) there were areas of tan or auburn hair and also, particularly in the pectoral region, scattered hairs tipped with dark brown.

Equilin. A dark grey-brown male that had absorbed 1.1 mg. in 121 days had scanty hair on his flanks and on the posterior half of his back. Another male and one female of the same strain are still carrying pellets at the time of writing (162 days after implantation). The male has scanty hair on the belly, and the female has scanty hair on the back and alopecic areas on the medial surfaces of the hind limbs. In the latter animal there are also curious dorsal tufts of short black hair.

Diethylstilbestrol methyl ether. Thirteen males and two females of the dark grey-brown strain carried pellets beyond the 35th day, the time at which hair changes were first observed. Of the 13 rats, 9 males and one female (65–150 days, 3.8–8.9 mg.) showed such changes. Four rats had scanty hair on various areas of the body; one of the four, a male, also had well developed, completely black mammary glands.

Another of the four rats, as well as four additional animals, had a curious triangular area, roughly 8×8×8 mm., postero-medial to one or both ears; the area was either completely hairless or else bore only a small tuft or two of short black hairs. The ninth rat, a male, had an extreme alopecia, being virtually hairless on the ventral thorax and the entire posterior half of the body. As in other alopecic animals, the skin showed no gross manifestation of disease.

Diethylstilbestrol methyl ether acetate. Hair changes began to appear in animals of the dark grey-brown strain about 100 days after they received pellets. Five males thus far autopsied (120–177 days, 6.0–8.2 mg.) since the end of the initial 100-day period showed scantiness of dorsal or ventral hair in three cases, while one of the four rats plus a fifth animal had a triangular area of complete alopecia postero-medial to the left ear. Of two males and one female still surviving at the time of writing (165 days after implantation of pellets), one male has made no response, while the remaining rats have scanty dorsal or ventral hair.

Diethylstilbestrol dipropionate. Implantation of pellets in rats of the dark grey-brown strain was followed by hair changes in the two females and in eight of the 13 males that carried pellets for 35 days or longer (40–145 days, 2.4–9.5 mg.). The hair changes were very similar to those observed in animals bearing diethylstilbestrol methyl ether pellets, ranging from scanty dorsal or ventral hair to one case of extreme alopecia in which the animal was virtually hairless on the ventral trunk and on the posterior half of the body. In two rats, small tufts of short black hairs were present in the alopecic areas.

Features of the hair changes associated with the absorption of all types of pellet were that, once established, the alopecia did not increase appreciably, that pigmentation of albino hair did increase progressively, and that the response of individual animals to a given type of pellet was highly variable, ranging from only slight hair loss to, in a few cases, alopecia covering more than half of the body or from slight to pronounced coloration of albino hair. Similarly, pigmentation of the pellet capsule or of the mammary gland occurred in only a few instances. There was no correlation in the majority of the cases between the amount of hormone absorbed and the degree of hair loss. There appeared to be no sexual difference in regard to hair changes, although relatively few females were used.

Hairs from a few albino rats were cleared in pine oil and examined microscopically. The yellow-brown pigment was present in the form of irregular masses, chiefly in the medulla, as was especially evident in those hairs which had split. No coloration appeared on the surface of the hairs.

The data on mammary gland growth in males, to which some reference has been made in the above paragraphs, will be more fully reported elsewhere.

DISCUSSION

The variation, independent of the length of treatment, in the response of both the albino and the dark grey-brown strain of rats to implantation of pellets of a given compound was marked, as has been indicated. The animals were not of carefully bred strains. In view of the well known genetic control of fur coloration, it seems probable that individual response to a given hormone was largely determined by the genetic constitution of the rat. The very different response of the albino and the dark grey-brown rats in those experiments in which both types received pellets of the same hormone exemplifies the influence of a major genetic difference on the reaction of the hair.

Hair changes were not noted in rats that received pellets of various additional

hormones (2) not mentioned in this report. It is possible in these experiments that either the periods of treatment (27–88 days) were too brief for a response to appear or that the hormones in question are incapable of causing hair changes in rats.

The implantation of ovaries and the injection of various estrogens into castrate and normal male and female guinea pigs has resulted in hyperpigmentation of the nipples, areolae, external genitalia, linea abdominalis, and abdominal skin (3–8). Unilateral local application of solutions of estrone or stilbestrol to the nipples of male guinea pigs resulted in increased pigmentation of the treated nipples (9). Hypogonad or castrate men and women receiving more or less extensive injections of folliculin, estrone, diethylstilbestrol, or testosterone propionate, the latter in conjunction with exposure to sun or ultraviolet light, are said to have responded by showing definite pigmentation in the various cases of nipples, areolae, linea abdominalis, and skin of the neck, body, and scrotum (10–16). It has frequently been suggested that the well known pigmentary changes in the nipples, areolae, and linea abdominalis of the pregnant woman are related to the increased estrogen content of the blood during pregnancy. No reference has been found in the literature to the appearance, following administration of a sex hormone to mammals, of pigment in the mammary gland or subcutaneous tissues.

The purpose of this report has been to describe certain experimental results without, at the present time, attempting to analyze the actual mechanisms responsible. Attention, however, should be called to the highly suggestive work of Figge and Allen on the rôle of estrone in melanin formation (17, 18).

SUMMARY

The subcutaneous implantation of pellets of crystalline androsterone, estrone, α-estradiol, α-estradiol benzoate, α-estradiol dipropionate, estriol, equilin, diethylstilbestrol methyl ether, diethylstilbestrol methyl ether acetate, and diethylstilbestrol dipropionate, but not of testosterone dipropionate, was usually followed after varying periods of time by partial pigmentation of the fur of albino rats and by alopecia in a strain of dark grey-brown rats. A few animals of both groups had deposits of black pigment in the pellet capsules or in the developed male mammary glands. An indication of the minimal treatment required to produce these effects is given.

REFERENCES

1. Chevrel-Bodin, L., D. Leroy and M. Cormier: *Bull. Soc. franc. de dermat. et syph.* 45: 458. 1938.
2. Forbes, T. R.: *Endocrinology* 29: 70. 1941, and unpublished results.
3. Lipschütz, A.: *Virchows Arch. f. path. Anat.* 276: 676. 1930.
4. Lipschütz, A., and M. Merino: *Compt. Rend. Soc. de Biol.* 111: 421. 1932.
5. Bloch, B., and A. Schrafl: *Arch. f. Dermat. u. Syph.* 165: 268. 1932.
6. Guldberg, G.: *Virchows Arch. f. path. Anat.* 294: 213. 1934.
7. Watrin, J.: *Compt. Rend. Soc. de Biol.* 115: 1455. 1934.
8. Tsubota, S.: *Jap. J. Dermat. & Urol.* 40: 14. 1936.
9. Fierz, F.: *Helvet. Chim. Acta* 22: 989. 1939.
10. Watrin, J.: *J. de méd. de Paris* 57: 869. 1937.
11. Hamilton, J. B., and G. Hubert: *Science* 88: 481. 1938.
12. Hamilton, J. B.: *Proc. Soc. Exp. Biol. & Med.* 40: 502. 1939.
13. Davis, M. E., and M. W. Boynton: *J. Clin. Endocrin.* 1: 339. 1941.
14. Byron, C. S., and P. Katzen: *J. Clin. Endocrin.* 1: 359. 1941.
15. Edwards, E. A., J. B. Hamilton, S. Q. Duntley and G. Hubert: *Endocrinology* 28: 119. 1941.
16. Lisser, H.: *Endocrinology* 27: 385. 1940.
17. Figge, F. H. J.: *Anat. Rec., Suppl.* 79: 21. 1941.
18. Figge, F. H. J. and E. Allen: *Endocrinology* 29: 262. 1941.

THE POTENCY OF STILBESTROL IN THE IMMATURE FEMALE RAT

HENRY M. LEE, E. B. ROBBINS, AND K. K. CHEN
From The Lilly Research Laboratories, Eli Lilly and Company
INDIANAPOLIS, INDIANA

SINCE THE DISCOVERY by Dodds, Golberg, Lawson, and Robinson (1) that stilbestrol (diethylstilbestrol or 4:4′-dihydroxy-α:β-diethylstilbene) possesses a marked degree of estrogenic action, several papers have appeared reporting the relative potency of stilbestrol to estrone. A survey of the literature as shown in table 1 reveals great differences of results, chiefly perhaps due to the different methods employed. In spayed mice, the two compounds were equally active when injected subcutaneously, but stilbestrol was superior when given orally. In spayed rats the ratio

TABLE 1. SURVEY OF THE LITERATURE ON THE RELATIVE ACTIVITY OF STILBESTROL AND ESTRONE

Author	Test Animal	Ratio of Stilbestrol to Estrone	
		By subcutaneous injection	By oral administration
Emmons (5, 6)	Spayed mouse	1	2.5
Leighty and Wick (8)	Spayed mouse	1	5.6
Sondern and Sealey (12)	Spayed mouse	1	65
Dodds, Golberg, Lawson, and Robinson (1)	Spayed rat	2–3	—
Dodds, Lawson, and Noble (2)	Spayed rat	2.5	—
Campbell, Dodds, Lawson, and Noble (4)	Spayed rat	2–3	50
Kreitmar and Sieckmann (9)	Spayed rat	5	20
Sondern and Sealey (12)	Spayed rat	1	80
Stasiak and Farkas (10)	Spayed rat	4.5	—
Koenig and Gustavson (11)	Spayed rat	1.48–3.16	—
Mazer, Israel, and Ravetz (13)	Spayed rat	5	20
Freud (7)	Spayed rat	4	—
von Haam, Hammel, Rardin and Schoene (14)	Immature female mouse	1	—
Grab (3)	Immature female rat	4	—
Huf (15)	Immature female rat	15	—
Sealey and Sondern (16)	Immature female rat	16	—

of activity of stilbestrol to estrone varied from 1 to 5 by subcutaneous administration, and that by mouth ranged from 20 to 80. When immature rats were used, two groups of workers (15, 16) obtained ratios as high as 15 to 16 following subcutaneous medication. No difference between stilbestrol and estrone was demonstrated in immature mice by subcutaneous injection (14).

Although there is no uniformity of the published data, as listed in table 1, one feature stands out prominently, that stilbestrol is relatively more active in spayed mice and rats by oral administration than by subcutaneous injection. In a preliminary report on stilbestrol from this laboratory (17), it was pointed out, however, that if a premature vaginal opening and an increase in the uterine weight of rats were used as criteria, stilbestrol was definitely more effective by subcutaneous injection than by

Received for publication December 5, 1941

TABLE 2. THE ACTION OF ESTRONE AND STILBESTROL ON VAGINAL INTROITUS AND UTERINE WEIGHT IN THE IMMATURE FEMALE RAT

Drug	Administration	Dose	Number of Open Vaginae/Number of Rats Used	Mean Uterine Weight
		μg. per rat		*mg.*
Estrone (laboratory specimen)	Oral	0.0[1]	0/15	19.6
		0.36[1]	0/10	21.0
		0.78[1]	0/10	21.7
		1.7	0/10	21.8
		3.0	0/5	35.4
		3.7	6/10	37.9
		5.3	1/5	41.4
		8.0	9/10	43.6
		9.5	3/5	47.2
		10.0	5/5	47.5
		16.8	5/5	56.5
		18.0	5/5	51.8
		30.0	5/5	67.8
		32.0	5/5	64.7
		56.0	5/5	62.5
		100.0[1]	5/5	53.4
Stilbestrol	Oral	0.004[1]	0/10	19.2
		0.013[1]	0/10	18.7
		0.04[1]	0/10	18.0
		0.13	0/10	25.2
		0.20	1/25	32.7
		0.28	6/25	38.4
		0.40	14/34	47.0
		0.50	5/5	63.1
		0.56	39/44	60.2
		0.64	5/5	68.9
		0.8	24/25	67.7
		1.2	5/5	76.5
		1.9	5/5	73.4
		3.0	5/5	80.0
Estrone (laboratory specimen)	Subcutaneous	0.28	0/3	23.3
		0.56	0/8	25.3
		0.76	0/5	27.0
		1.07	0/10	24.8
		1.5	1/10	34.8
		2.1	2/10	39.4
		2.94	3/10	37.4
		4.12	5/10	48.1
		5.77	7/10	46.7
		8.1	10/10	49.2
		11.3	10/10	53.6
		15.8	10/10	55.9
Estrone (International Standard)	Subcutaneous	2.1	1/8	42.7
		2.94	2/8	42.3
		4.12	4/8	50.5
		5.77	7/8	57.3
		8.1	7/8	63.9
Stilbestrol	Subcutaneous	0.03	0/10	24.7
		0.075	0/10	37.4
		0.12	9/18	50.7
		0.135	9/10	66.9
		0.15	7/8	64.6
		0.18	16/18	63.1
		0.21	7/8	69.2
		0.26	17/18	72.3
		0.28	3/3	79.5
		0.37	10/10	76.5
		0.56	13/13	83.4
		0.77	10/10	83.7
		1.05	5/5	81.5

[1] Not used in the calculation of the regression lines.

TABLE 3. COMPARISON OF THE ACTIVITY OF STILBESTROL AND ESTRONE ON THE BASIS OF VAGINAL INTROITUS

Administration	Estrogen	Median Introitus Dose ± Standard Error	Ratio of Potency Stilbestrol: Estrone
		μg. per rat	
Subcutaneous	Estrone (laboratory specimen)	3.656±0.378	31.9±3.2
	Estrone (International Standard)	3.941±0.476	
	Estrone (combined)	3.904±0.285	
	Stilbestrol	0.1225±0.0087	
Oral	Estrone (laboratory specimen)	4.629±0.704	11.9±1.9
	Stilbestrol	0.3904±0.0198	

mouth. Results obtained from a large series of experiments, presented in the following sections, substantiate this conclusion. Furthermore, the ratio of activity between stilbestrol and estrone in immature rats may be a better measure for the determination of a therapeutic dose than the ratio in spayed rats.

EXPERIMENTAL PROCEDURE

Two samples of estrone were employed, one being the international standard and the other a laboratory specimen. Only one sample of stilbestrol of highest purity was examined. Both estrogens were dissolved in cottonseed oil, and appropriate dilutions were made with the same solvent. Groups of immature, female albino rats, aged 21 to 23 days, were given, orally or subcutaneously, various amounts of stilbestrol or estrone, divided into 3 equal doses and administered on 3 successive days. The total dose of estrone varied from 0.36 to 100 micrograms and that of stilbestrol, from 0.004 to 3 micrograms, per rat. On the fifth day, or approximately 96 hours after the initial dose, the animals were examined for vaginal opening (introitus) and then autopsied. The uterus of each rat was rapidly but carefully dissected from the oviduct-uterine junction to the cervix, free from mesometrium. After the isolated organ was blotted between sheets of filter paper, it was quickly weighed on a torsion balance which was accurate to 0.1 mg. In order to increase the accuracy of observation, simultaneous assays of stilbestrol and estrone were made with each mode of administration, oral or subcutaneous.

RESULTS

All of the observations recorded with both stilbestrol and estrone are summarized in table 2. The mean uterine weight is the average of the weights of the organs of each group of rats. Since the response of premature vaginal opening follows an all-or-none fashion, the data can be considered in the same manner as the dosage-mortality results. Median introitus doses can be satisfactorily computed by the method of Bliss (18) as given in table 3. It should be noted that the data with the laboratory specimen and international standard of estrone check each other, for their difference is not statistically significant. When the results of the two series are combined, the median introitus

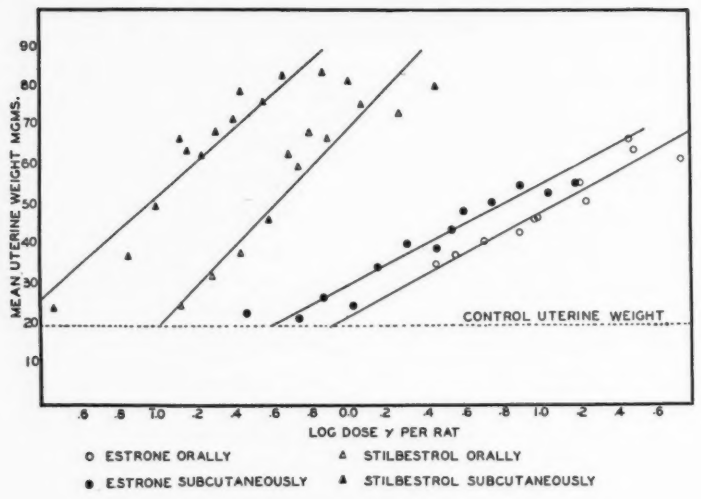


Fig. 1. ACTION OF STILBESTROL AND ESTRONE ON UTERINE WEIGHT OF THE IMMATURE FEMALE RAT.

dose then becomes $3.904 \pm 0.285\mu$g. per rat. The ratio of potency of stilbestrol to estrone based on this figure is approximately 32:1 by subcutaneous injection. If the effects of the two drugs orally were compared the ratio of activity between stilbestrol and estrone would be 12:1 as shown in table 3. These ratios cannot be considered radically different from those previously reported (17), since "t" tests show that the differences are not significant statistically.

Regarding the hypertrophy of the uterus, a similar relationship of the two products occurs. This effect has been utilized by other investigators for the comparison of activity of estrogenic substances (14–16, 19–24). An analysis of variance of the uterine weight of our animals which might have been attributed to differences in body weight was made according to Fisher's method (25). It was found that variation in body weight throughout the range observed (28 to 45 gm.) had no appreciable effect upon the final uterine weight. It is thus possible to plot the final mean uterine weight against the logarithm dose and to calculate regression lines from the data by the method of least squares, as illustrated in figure 1. By inspection, it is apparent that the curves of estrone are not parallel to those of stilbestrol. The ratio of potency of stilbestrol to estrone, therefore, will vary according to the level that is chosen for comparison. However, if the mean uterine weight is arbitrarily selected as the point of comparison, the ratio of potency of stilbestrol to estrone will be approximately 8:1 by mouth and 22:1 subcutaneously. In other words, the incidence of vaginal opening and changes in the uterine weight follow a relatively similar course. The results in both instances indicate that stilbestrol as compared with estrone is definitely more effective by subcutaneous injection than by mouth under the conditions set forth in these experiments.

DISCUSSION

The results obtained in this study bring about two points which may deserve emphasis. In the first place, the relative potency of stilbestrol to estrone varies according to the method of assay. In spayed rats, as reported by many workers, the ratio of activ-

ity of stilbestrol to estrone lies between 2:1 and 5:1. By mouth, the ratio becomes much higher. In immature rats as used in our investigations, however, this order is reversed. The ratio of activity by subcutaneous injection is approximately 32:1 and by mouth, 12:1, provided the vaginal opening is used as the criterion. Secondly, the ratio of activity based on the response of immature rats could have been a better guide for determination of a therapeutic dose for humans than that based on studies in spayed rats. For example, by employing daily doses of 2 to 6 mg. as recommended by the British workers (26, 27), the American clinicians observed toxic reactions, chiefly nausea and vomiting, in as high as 60 to 80 per cent of their patients (28–31). Had they been aware of the ratio of 12:1 in immature rats by mouth, they might have started with smaller therapeutic doses. It was only by caution and experience that the daily dose was finally reduced to 0.5 to 1 mg. or less—with a minimum of untoward symptoms (32–34).

Notwithstanding, the production of nausea and vomiting appear to be the inherent properties of stilbestrol. The symptoms occur, or become aggravated, when excessive doses are used. It was this unfortunate circumstance which enhanced the difficulty of clinical investigations at the beginning.

SUMMARY

Using the response of the vaginal introitus of immature female rats as the criterion stilbestrol is shown to be approximately 12 times as active as estrone by oral administration, and 32 times as active when injected subcutaneously.

The increase in uterine weight of the same animals follows a similar course as the vaginal opening, but the results are less suitable for a comparison of the responses to stilbestrol and estrone.

REFERENCES

1. Dodds, E. C., L. Golberg, W. Lawson and R. Robinson: *Nature* 141: 247. 1938.
2. Dodds, E. C., W. Lawson and R. L. Noble: *Lancet* 1: 1389. 1938.
3. Grab, W.: *München. med. Wchnschr.* 86: 436. 1939.
4. Campbell, N. R., E. C. Dodds, W. Lawson and R. L. Noble: *Lancet* 2: 312. 1939.
5. Emmons, C. W.: *J. Physiol.* 94: 22P. 1939.
6. Emmons, C. W.: *J. Endocrinol.* 1: 142. 1939.
7. Freud, J.: *Acta brev. Neerland.* 9: 11. 1939.
8. Leighty, J. A., and H. J. Wick: *Endocrinology* 25: 597. 1939.
9. Kreitmar, H., and W. Sieckmann: *Klin. Wchnschr.* 18: 156. 1939.
10. Stasiak, A., and E. Farkas: *Arch. d. Pharm.* 278: 176. 1940.
11. Koenig, V. L., and R. G. Gustavson: *J. Pharmacol. & Exper. Therap.* 69: 355. 1940.
12. Sondern, C. W., and J. L. Sealey: *Endocrinology* 27: 670. 1940.
13. Mazer, C., S. L. Israel and E. Ravetz: *J.A.M.A.* 116: 675. 1941.
14. von Haam, E., M. A. Hammel, T. E. Rardin and R. H. Schoene: *Endocrinology* 28: 263. 1941.
15. Huf, E.: *Arch. f. exper. Path. u. Pharmakol.* 197: 415. 1941.
16. Sealey, J. L., and C. W. Sondern: *Endocrinology* 29: 356. 1941.
17. Chen, K. K.: *Quart. Bull. Indiana Med. Cent.* 3: 15. 1941.
18. Bliss, C. I.: *Quart. J. Pharm. & Pharmacol.* 11: 192. 1938.
19. Bülbring, E., and J. H. Burn: *J. Physiol.* 85: 320. 1935.
20. Marlow, H. W.: *Science* 84: 377. 1936.
21. Dorfman, R. I., T. F. Gallagher and F. C. Koch: *Endocrinology* 19: 33. 1935.
22. David, K., and S. E. de Jongh: *Biochem. J.* 29: 371. 1935.
23. Astwood, E. B.: *Endocrinology* 23: 25. 1938.
24. Lauson, H. D., C. G. Heller, J. B. Golden and E. L. Sevringhaus: *Endocrinology* 24: 35. 1939.
25. Fisher, R. A.: Statistical Methods for Research Workers. Oliver & Boyd, London, 3d ed., 1930.
26. Kellar, R. J., and J. K. Sutherland: *J. Obst. & Gynaec. Brit. Emp.* 41: 1. 1939.
27. Winterton, W. R., and T. N. MacGregor: *Brit. M. J.* 1: 10. 1939.
28. Shorr, E., F. H. Robinson and G. Papanicolaou: *J.A.M.A.* 113: 2312. 1939.
29. Collins, C. G., J. C. Weed, B. B. Weinstein and F. R. Lock: *Am. J. Obst. & Gynec.* 39: 117. 1940.
30. Diddle, A. W., and W. C. Keettel: *Am. J. Obst. & Gynec.* 39: 791. 1940.
31. Weed, J. C., B. B. Weinstein, F. R. Lock, T. W. Douglas and C. G. Collins: *Am. J. Obst. & Gynec.* 39: 1047. 1940.
32. Sevringhaus, E. L.: *J.A.M.A.* 114: 685. 1940.
33. MacBryde, C. M., D. Castrodale, E. Loeffel and H. Freedman: *J.A.M.A.* 117: 1240. 1941.
34. Peelen, J. W.: *J. Michigan M. Soc.* 40: 873. 1941.

THE INFLUENCE OF STILBESTROL AND THYROXIN ON GALACTOSE ABSORPTION AND LIVER FUNCTION

ROBERT C. GRAUER, WILLIAM F. STARKEY
AND ELEANOR SAIER

From the Department of Research in Endocrinology and Metabolism, William H. Singer Memorial Research Laboratory of the Allegheny General Hospital
PITTSBURGH, PENNSYLVANIA

PRELIMINARY TO ITS USE IN THE STUDY of the thyrotoxic state in the human, the galactose tolerance test, as originally advocated by Althausen (1) was employed as a unit of measurement in dogs. Following Althausen's report, Maclagan and Rundle (2) as well as King et al. (3, 4) employed the same type of test but they placed a different interpretation on their findings. Briefly, the American authors advanced the explanation that the thyroid hormone increases the rate of absorption of galactose from the intestinal tract (5). In support of this concept, they quoted the work of Soskin et al. (6). The English authors (2, 3, 4) contended that the increase of the galactose in the circulating blood in the thyrotoxic state was due to liver damage; that the blood was therefore not cleared sufficiently rapidly of the galactose which was absorbed from the intestinal tract. Maclagan and Rundle (2) quoted the literature (7, 8) in which evidence was advanced that liver damage was frequently associated with thyrotoxicosis. King et al. (4) produced liver damage in rabbits with carbon tetrachloride and found that the damaged liver interfered with the utilization of the intravenously injected galactose. Both the American and the English investigators agreed that the test was a valuable aid in making a diagnosis of the thyrotoxic state. The difference appeared to be largely in the interpretation of the results that were obtained.

While conducting investigations of patients in whom thyrotoxicosis existed, large doses of the synthetic estrogenic substance diethylstilbestrol were employed in the preparation for operation. The galactose tolerance test, among others, was used during various phases of this investigation. In order to explain the results that were obtained and to help elucidate the mechanism of action of galactose under these conditions, experimental studies were carried out on 12 dogs. This report deals with the results of this study.

METHOD OF STUDY

Twelve adult dogs (5 males and 7 females) were employed in the investigation. They had been kept on a constant diet of Ubico Dog Chow since puppyhood and were raised in our own laboratory. In order to establish control curves, oral and intravenous galactose tolerance tests were performed on each dog, after fasting, and previous to the administration of medication. A 50 per cent solution of galactose in dosage of 0.5 gm. per kg. of body weight was administered to each animal. The oral galactose was introduced into the stomach by means of a tube. A fasting sample of venous blood was taken and subsequent samples of blood were drawn at intervals of

Received for publication December 3, 1941.

30, 60 and 90 minutes. The blood galactose determinations were then performed in accordance with Raymond and Blanco's modification of the Somogyi method as employed by Althausen (9). Two days later determinations were made on the same dogs, using the intravenous route of galactose administration. Four dogs (*No.* 700, 704, 706, and 710), which were employed subsequently, had no preliminary galactose tolerance tests performed. The averages of the other 8 dogs were used as normal controls and curves were plotted (fig. 1). The results of these determinations at

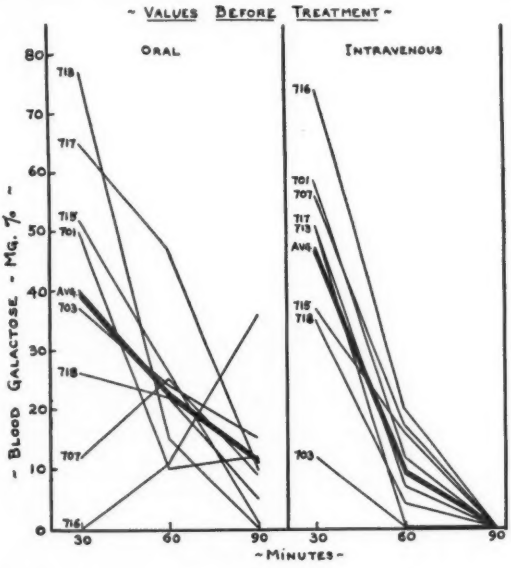


Fig. 1. GLUCOSE TOLERANCE CURVES OF DOGS THAT RECEIVED GALACTOSE BEFORE TREATMENT BY THE ORAL AND INTRAVENOUS ROUTE. Dark line indicates average of all values and shows the trend.

the various time intervals are listed in table 1. The 'galactose index' refers to the sum of the values at 30, 60 and 90 minutes (2).

Following the establishment of normal curves, 4 dogs (*No.* 701, 717, 718, and 707) were given daily intramuscular injections of 0.5 mg. of thyroxin[1] per kg. of body weight. Oral galactose tolerance tests were made at intervals of 7, 14 and 20 days and were repeated intravenously at intervals of 9, 16 and 22 days.

Four dogs (*No.* 713, 706, 710, and 715) received daily injections of 5 mg. of diethylstilbestrol[2] in oil. Four dogs (*No.* 704, 700, 716, and 703) received daily injections of 10 mg. of diethylstilbestrol in oil. Both of these groups received the injections daily for a period of 22 days. Oral and intravenous galactose tolerance tests were made at the end of this time. Four dogs were selected from this group (*No.* 706, 713, 703, and 716) and these were given simultaneous injections of thyroxin and stilbestrol for 6 succeeding days. The galactose tolerance tests were repeated again at the end of this time. Curves comparing the oral and intravenous routes were plotted for each of the groups.

[1] This material was supplied to us by the Roche-Organon Company, Nutley, N. J. through the kindness of Dr. R. D. Shaner.

[2] This material was kindly supplied by E. R. Squibb & Sons, New York City.

At the end of the course of treatment, the dogs were anesthetized by intravenous injection of nembutal and a biopsy of the liver was taken from each. Control biopsies were taken from 4 untreated dogs. The material thus obtained was fixed in Zenker's fluid and stained with phloxine and methylene-blue for microscopic examination.

RESULTS

The oral administration of galactose in 8 dogs revealed that peaks in the blood sugar are reached at 30 minutes and at 60 minutes. The rate of absorption varied in some of the dogs, a phenomenon that also has been observed in the human (10). A composite average of all these determinations is shown in figure 1 by the heavy black line. The numerical values of these are indicated in table 1. The average galactose index for all dogs was 73. When the galactose was introduced intravenously, a much more constant trend was observed (fig. 1, table 1). The 30-minute value compared favorably with that of the same period after oral administration of the galactose, but the 60-minute value was lower; all galactose was eliminated in 90 minutes in 5 of the 8 dogs. The average galactose index by this route was found to be 56.5.

TABLE 1. GALACTOSE TOLERANCE TESTS IN DOGS BEFORE TREATMENT

Blood galactose values, in mg. per 100 cc.

Dog No.	Sex	Wt.	Oral Galactose				Intravenous Galactose			
			Time in minutes			Galactose index	Time in minutes			Galactose index
			30	60	90		30	60	90	
		lb.	mg. %	mg. %	mg. %		mg. %	mg. %	mg. %	
713	M	23	77	15	0	92	51	0	0	51
715	F	30	52	26	1	79	37	16	0	53
703	F	23	37	24	15	76	12	0	0	12
716	F	30	0	11	36	47	74	20	0	94
717	F	27	65	47	10	122	51	7	0	58
701	F	27	50	10	12	72	58	12	0	70
718	F	33	26	22	5	53	35	4	0	39
707	M	30	12	25	9	46	56	17	0	73
Average			40	22	11	73	47	9	0	56
700	M	40	No determinations before treatment							
704	M	40								
706	M	40								
710	F	17								

When the galactose tolerance tests were performed after the dogs had received stilbestrol for 22 days, it was observed that the blood sugar values were considerably higher by the oral route than were the values for the average before treatment. These are shown in figure 2 and in table 2. The broken line indicates the curves that were obtained from dogs that received 10 mg. of stilbestrol, while the solid lines represent the values that were obtained for dogs that received 5 mg. of stilbestrol. The average galactose indices by the oral route were 105 and 135, respectively, while the values for the intravenous route were 35 for each of the two groups. It appears that stilbestrol greatly increases the rate of absorption of galactose from the intestinal tract and at the same time hastens galactose clearance from the blood, as indicated by the low values after intravenous administration. The dogs that received 10 mg. of stilbestrol showed an average value at 60 minutes of 0.25 as compared to the average value of 9.5 mg.% in the dogs that were untreated.

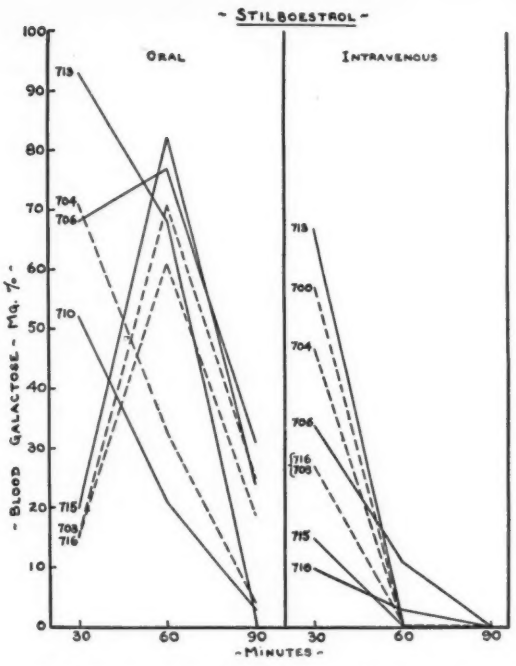


Fig. 2. DAILY ADMINISTRATION OF 5 MG. (*solid line*) AND 10 MG. (*broken line*) OF STILBESTROL FOR 22 DAYS. Marked absorption from intestinal tract (oral) and rapid utilization when injected intravenously at end of treatment.

TABLE 2. GALACTOSE TOLERANCE IN DOGS AFTER TREATMENT WITH DIETHYLSTILBESTROL

Blood galactose values, in mg. per 100 cc.

Dog No.	Sex	Weight lb.	Oral Galactose				Intravenous Galactose			
			Time in minutes			Galactose index	Time in minutes			Galactose index
			30	60	90		30	60	90	
Diethylstilbestrol, 5 mg./day										
713	M	23	93	68	1	162	67	0	0	67
706	M	40	68	77	31	176	34	11	0	45
710	F	17	52	21	3	76	10	3	0	13
715	F	30	20	82	24	126	15	0	0	15
Average			58	62	15	135	31	3	0	35
Diethylstilbestrol, 10 mg./day										
704	M	40	71	33	4	108	47	0	0	47
700	M	40					57	1	0	58
716	F	30	15	71	25	111	27	0	0	27
703	F	23	16	61	19	96	27	0	0	27
Average			34	55	16	105	39	0.25	0	40

Four dogs that received daily intramuscular injections of thyroxin showed an average loss of weight of 4 pounds at the end of 7 days. The dogs were nervous, irritable, overactive and showed the behavior commonly associated with the experimental thyrotoxic state. When the galactose tolerance tests were repeated at the end of 7 days of treatment, the animals showed a rapid rise in the galactose values by the oral route of administration. The peaks were reached at 30 and 60 minutes and there was a definite rise at the end of 90 minutes (table 3). The average galactose index was

TABLE 3. GALACTOSE TOLERANCE IN THYROXIN-TREATED ANIMALS

Blood galactose values, in mg. per 100 cc.

Dog No.	Sex	Wt. lb.	Wt. loss lb.	Oral Galactose				Intravenous Galactose			
				Time in minutes			Galactose index	Time in minutes			Galactose index
				30	60	90		30	60	90	
Thyroxin, 7 days								*Thyroxin, 9 days*			
701	F	27	3.5	45	26	19	90	42	4	0	46
717	F	27	4	84	48	11	143	32	5	0	37
718	F	33	3	53	68	36	157	51	12	0	63
707	M	30	6	26	51	36	113	56	9	0	65
Average			4	52	48	25	126	45	7	0	52
Thyroxin, 14 days								*Thyroxin, 16 days*			
701				10	10	0	20	41	3	0	44
717				41	20	3	64	84	25	6	115
718				0	3	5	8	69	40	3	112
707				0	4	17	21	38	3	0	41
Average				13	9	6	28	58	18	2	78
Thyroxin, 20 days								*Thyroxin, 22 days*			
701				31	28	4	63	52	27	0	79
717				43	16	0	59	71	31	0	102
718				21	29	15	65	82	19	0	101
707				22	16	7	45	72	19	0	91
Average				29	22	6	58	69	26	0	93

126. When the same tests were repeated at the end of 14 days, we observed that the values dropped considerably, so that the average galactose index was only 28. At the end of 20 days of treatment, the values again began to rise and approached normal values at the 60-minute period. The galactose index was then 58. Two days were allowed to elapse between the oral and intravenous determinations. The intravenous galactose tolerance test at the end of 9 days of thyroxin administration showed no marked deviation from the pretreatment level. At the end of 16 days, the values began to rise above those obtained before treatment, the galactose index being 76 compared to the control of 56.5. At the end of 22 days, the index rose to 93. These results are graphically illustrated in figure 3. It appears that an early rapid absorption from the intestinal tract occurs during the first week of treatment with thyroxin and that delay in clearing the blood of the galactose occurs later when there probably is interference with hepatic utilization of the carbohydrate.

Four dogs that had been receiving stilbestrol for 22 days then received daily simultaneous injections of thyroxin for 6 succeeding days. The behavior of these dogs was in striking contrast to that of those receiving thyroxin alone. There were no outward signs of nervous stimulation, such as irritability, snapping or hyperactivity. It appeared as though the stilbestrol had a quieting effect on the sympathetic nervous stimulation which was seen when thyroxin alone was administered. Reference to

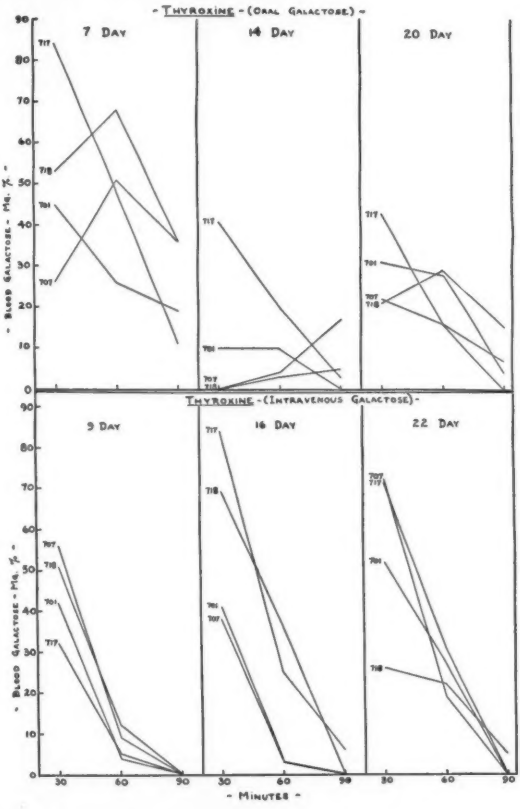


Fig. 3. TYPE OF CURVE PRODUCED BY THYROXIN ADMINISTRATION; early rapid absorption from intestinal tract; later, decreased utilization by liver due to liver damage. For the 22-day period (intravenous galactose) the 30-minute point for *dog 718* should begin at 82 mg. %.

table 4 and figure 4 shows that the galactose index was increased after administration by both the oral and the intravenous route. With the exception of the oral values in the dogs that received 10 mg. of stilbestrol, all other values were considerably higher than normal. The implication of the results that were obtained with this combination is that we are dealing with a combined factor of an increased rate of absorption plus a delay in clearance from the blood, indicating possible interference with normal hepatic utilization of the sugar.

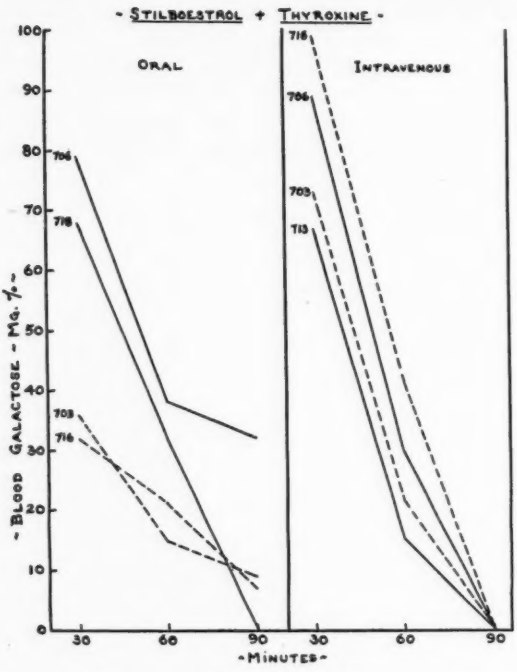


Fig. 4. CURVE DEMONSTRATING THAT A COMBINATION OF THYROXIN AND STILBESTROL PRODUCES RAPID ABSORPTION OF THE GALACTOSE AND A DELAYED UTILIZATION BY THE LIVER

Biopsies of the liver showed the cells to have the normal appearance of vacuolation which has been interpreted as due to glycogen in the liver. The central veins and periportal tissue showed no abnormalities. The sinusoids were of normal caliber. The

TABLE 4. GALACTOSE TOLERANCE IN THYROXIN AND DIETHYLSTILBESTROL-TREATED ANIMALS
Blood galactose values, in mg. per 100 cc.

Dog No.	Sex	Wt. lb.	Oral Galactose				Intravenous Galactose			
			Time in minutes			Galactose index	Time in minutes			Galactose index
			30	60	90		30	60	90	
Thyroxin and 5 mg. of diethylstilbestrol										
706	M	40	79	38	32	149	89	30	0	119
713	M	23	68	32	0	100	67	15	0	82
Average			73	35	16	124	78	22	0	100
Thyroxin and 10 mg. of diethylstilbestrol										
703	F	23	36	15	9	60	73	22	0	95
716	F	30	32	21	7	60	99	42	0	141
Average			34	18	8	60	86	32	0	118

sections that were examined from dogs that had received thyroxin for 22 days showed a loss in vacuolation, implying glycogen depletion. This was especially marked in *dog 707*. The sinusoids were wider owing to the shrinking of the liver cells. There was some evidence of early cellular break-down. Sections taken from *dogs 701, 717* and *718* showed the same reaction in varying degrees. The dogs that had received stilbestrol alone, revealed some loss of vacuolation which was intermediary between that seen in the normal controls and those that had received thyroxin for 22 days. The glycogen depletion was more marked around the periportal areas than around the central veins. Three of 4 dogs (*No. 710, 704,* and *700*) showed mild polymorphonuclear infiltration about the central veins. Section taken from *dog 706*, which had received thyroxin plus 5 mg. of stilbestrol, revealed a total loss of glycogen and resembled the sections of the liver of the dogs that received thyroxin alone for a period of 22 days. It should be observed that this dog had had the highest galactose index both by the oral and intravenous route when compared to the other dogs of the same group.

DISCUSSION

From the results that were obtained in this study, it appears that both factors, namely, the increased rate of intestinal absorption and the degree of liver damage, play a part in the explanation of the type of modified galactose tolerance curve that is obtained by virtue of the action of the thyroid hormone. After 7 days of administration of thyroxin, the orally administered galactose is rapidly absorbed and gives increased values in the blood while the intravenous values are within normal range. This would indicate that the early effect of thyroxin is to increase the rate of absorption of galactose while the intravenous values imply that liver damage had not yet supervened. With the continued use of thyroxin, disturbance in liver function occurs and the intravenous galactose values rise, reaching an increase of 60 per cent above the normal in 22 days. Liver glycogen is reduced by thyroxin (11, 12); consequently, in order to replace the depleted liver glycogen, the utilization of the galactose by the liver is heightened before liver damage occurs. This probably explains the differences that are obtained by the oral and the intravenous routes.

The type of curve secured when stilbestrol alone was used can be explained on the basis that stilbestrol is diabetogenic, as was indicated by Ingle (13). It was previously shown by Janes and Nelson (14) that stilbestrol produced glyconeogenesis and that the liver glycogen in rats that received 0.1 mg. of stilbestrol daily, rose from 75 mg. per cent to 409 mg. per cent in 20 days. From this we can postulate that the stilbestrol in the dogs hastened glyconeogenesis with a consequent increase in utilization of the available administered galactose, producing a rapid absorption of the sugar from the intestinal tract and a still more rapid clearance from the blood due to the specific avidity of the liver for that sugar. Since we have shown that stilbestrol, when administered alone, did not produce demonstrable liver damage (15) the manner in which it affected galactose absorption differed from thyroxin, which ultimately did produce liver damage. Thyroxin, early in its administration, caused an increased absorption of galactose from the intestinal tract and a normal rate of clearance from the blood. Later (after 16–22 days of treatment) the galactose clearance from the blood was delayed due to liver damage, producing increased blood galactose values. When both thyroxin and stilbestrol were employed, a combined effect resulted which was reflected in increased oral and intravenous values.

Microscopic findings in the liver sections were in keeping with the functional capacity of the liver, as evidenced by the values of the blood galactose when given orally and compared to those obtained by the intravenous route.

CONCLUSIONS

Daily injections of thyroxin appear to increase the rate of absorption of galactose from the intestinal tracts of dogs during the first 7 days. This ability is gradually decreased after 14 days and is regained in 20 days.

Thyroxin given over a long period of time, 16 to 22 days, produces liver damage with a resulting increase in the galactose values in the blood.

Stilbestrol increases the rate of absorption of galactose from the intestinal tract and hastens its clearance from the blood. The glyconeogenic function of stilbestrol in this connection is postulated.

Stilbestrol plus thyroxin causes an increased rate of absorption and a delay in the clearance of galactose from the blood.

The findings in an histologic examination of liver biopsies were in accord with the results reported.

REFERENCES

1. Althausen, T. L., and G. K. Wever: *J. Clin. Investigation* 16: 257. 1936.
2. Maclagan, N. F., and F. F. Rundle: *Quart. J. Med.* 9: 215. 1940.
3. King, E. J., and R. S. Aitken: *Lancet* 2: 543. 1940.
4. King, E. J., C. V. Harrison and G. E. Delroy: *Lancet* 2: 541. 1940.
5. Althausen, T. L.: *J. Clin. Investigation* 16: 658. 1937.
6. Soskin, S., R. Levine and R. E. Heller: *Am. J. Physiol.* 125: 220. 1938.
7. Youmans, J. B., and L. M. Warfield: *Arch. Int. Med.* 37: 1. 1926.
8. Beaver, D. C., and J. de J. Pemberton: *Ann. Int. Med.* 7:543. 1933.
9. Althausen, T. L., J. C. Lockhart and M. H. Soley: *Am. J. M. Sc.* 199: 342. 1940.
10. Grauer, R. C., and C. W. W. Elkin: Unpublished data.
11. Coggeshall, H. C., and J. A. Greene: *Am. J. Physiol.* 105: 103. 1933.
12. Frazier, W. D., and H. Frieman: *Surg., Gynec. & Obst.* 60: 27. 1935.
13. Ingle, D. J.: *Endocrinology* 29: 838. 1941.
14. Janes, R. G., and W. O. Nelson: *Proc. Soc. Exper. Biol. & Med.* 43: 340. 1940.
15. Grauer, R. C., and N. Nugent: *Surg., Gynec. & Obst.* In press.

LACK OF EFFECT OF THYROXIN ON BLOOD SUGAR AND GLYCOGEN STORES OF FASTED HYPOPHYSECTOMIZED RATS[1]

VIRGIL V. HERRING, HEINZ FRAENKEL-CONRAT AND HERBERT M. EVANS

From the Institute of Experimental Biology, University of California
BERKELEY, CALIFORNIA

THE MAINTENANCE during fasting of normal blood sugar and glycogen stores is dependent upon the gluconeogenetic mechanism. In hypophysectomized animals this mechanism appears to be impaired; at any rate, there is a rapid fall in all carbohydrate levels when such animals are fasted. While it has been shown that the hypophyseal control of sugar formation and utilization is mediated primarily by the adrenal cortex (1), there is evidence also for other mechanisms. Thus the pituitary appears to have another, probably direct, effect on the maintenance of muscle glycogen (2). On the possible rôle of the thyroid in regard to gluconeogenesis no general agreement has been reached. Russel's findings in hypophysectomized rats (3), as well as those of Dohan and Lukens (4, 5) in depancreatized animals do not suggest that the thyroid is similarly active as is the adrenal cortex in promoting gluconeogenesis. On the other hand, Wells and Kendall (6, 7) have stated that in phloridzinized rats the effect of hypophysectomy in decreasing the glycosuria can be attributed to the atrophy of both adrenal cortex and thyroid and that only by simultaneous administration of both thyroxin and certain cortical hormones can the rate of gluconeogenesis and thus of the sugar excretion be restored to that of the unoperated phloridzinized rat. Similarly, the finding of Soskin (8) that thyroxin enables hypophysectomized dogs to maintain normal blood sugar levels during fasting favors the assumption that this hormone may stimulate gluconeogenesis.

It was therefore decided to reinvestigate the effect of thyroxin on the blood sugar and glycogen stores of fasting hypophysectomized rats. In two experiments male rats, 65 to 90 days old and 2 to 4 weeks postoperative were injected subcutaneously with 0.015 mg. of thyroxin daily. Similar groups of 5 to 9 rats served as controls. After 10 days of injection, all rats were fasted for 24 hours preceding autopsy; they were anesthetized with amytal and the removal of samples and the analytical procedures carried out immediately. As table 1 shows, thyroxin had no effect in preventing the fall in blood sugar and the exhaustion of liver and muscle glycogen stores.[2] It must therefore be concluded that this dose of thyroxin either does not stimulate gluconeogenesis in hypophysectomized rats, or that any such action is balanced by the increased utilization of carbohydrate provoked by the hormone. This dose of thyroxin

Received for publication December 29, 1941.

[1] Aided by grants from the Board of Research of the University of California and the Rockefeller Foundation, New York City, and Parke, Davis and Company, Detroit, Michigan. We wish to acknowledge assistance from the Works Projects Administration, Project No. OP-65-1-08, Unit A-5.

[2] Similarly ineffective was a preparation of purified pituitary thyrotrophic hormone (9) which was administered to similar rats at an equivalent dose (0.4 mg. or 15–20 chick units daily) (10); it caused pronounced hypertrophy of the thyroid and increase in liver weight.

was sufficient to raise the oxygen consumption to normal levels and caused the liver-weight increase characteristic for this hormone (10).

Besides these experiments, blood sugars were determined also in two groups of hypophysectomized male and female rats (4–6 weeks postoperatively) after a fast of only 5 to 6 hours. Untreated rats showed blood sugar values averaging 109 and 120 mg. per cent, while those treated with thyroxin (approximately 10 micrograms per 100 gm. of body weight daily) for 21 or 17 days showed values averaging 73 and 87 mg

TABLE 1. LACK OF EFFECT OF THYROXIN ON BLOOD SUGAR AND GLYCOGEN STORES OF FASTED HYPOPHYSECTOMIZED RATS[1]

	No. of Rats	Body Weight		Blood Sugar	Liver Glycogen	Muscle Glycogen	Liver in % of Body Wt.	O_2 Consumption
		At onset	Change					
		gm.	gm.	mg. %	%	mg. %		l./sq. m./day
Treated	5	211	−5	35	0.16	344	3.9	
Control	9	198	−3	44	0.12	397	3.2	
Treated	6	137	−3	56	0.19	485	3.8	166
Control	6	144	−6	54	0.18	513	3.0	124

[1] Male rats, 2 to 4 weeks p.o. at onset of experiment; injected for 10 days (0.015 mg. of thyroxin daily); fasted for 24 hours preceding autopsy.

per cent respectively. The fact that thyroxin actually appears to accentuate the hypoglycemic tendency of hypophysectomized rats may be due to its stimulation of the metabolic rate which in these experiments was found similar to that of normal rats. It may also be due to its action on the pancreas, which will be discussed in the following paper.

SUMMARY

Neither pituitary thyrotropic hormone nor thyroxin, when administered to hypophysectomized rats for 10 days, prevented the rapid appearance of hypoglycemia and the exhaustion of glycogen stores during fasting.

REFERENCES

1. LONG, C. N. H., B. KATZIN AND E. G. FRY: *Endocrinology* 26: 309. 1940.
2. RUSSEL, J. A., AND L. L. BENNET: *Proc. Soc. Exper. Biol. & Med.* 34: 279. 1936.
3. RUSSEL, J. A.: *Am. J. Physiol.* 122: 547. 1938.
4. DOHAN, F. C., AND F. D. W. LUKENS: *Am. J. Physiol.* 122: 367. 1938.
5. LUKENS, F. D. W., AND F. C. DOHAN: *Am. J. Physiol.* 129: 408. 1940.
6. WELLS, B. B., AND E. C. KENDALL: *Proc. Staff Meet., Mayo Clin.* 15: 493. 1940.
7. WELLS, B. B., AND A. CHAPMAN: *Proc. Staff Meet., Mayo Clin.* 15: 503. 1940.
8. SOSKIN, S., R. LEVINE AND R. E. HELLER: *Am. J. Physiol.* 125: 220. 1939.
9. FRAENKEL-CONRAT, J., H. L. FRAENKEL-CONRAT, M. E. SIMPSON AND H. M. EVANS: *J. Biol. Chem.* 135: 199. 1940.
10. FRAENKEL-CONRAT, H. L., M. E. SIMPSON AND H. M. EVANS: *Am. J. Physiol.* January, 1942. In press.

EFFECT OF THYROXIN ON THE INSULIN CONTENT OF THE RAT'S PANCREAS[1]

HEINZ FRAENKEL-CONRAT, VIRGIL V. HERRING, M. E. SIMPSON AND HERBERT M. EVANS

From the Institute of Experimental Biology, University of California
BERKELEY, CALIFORNIA

WITHIN RECENT YEARS various hormones have been found to affect the insulin content of the rat's pancreas. Marks, Young and coworkers, as well as Soong, have clearly demonstrated that pituitary extracts may increase pancreatic insulin (1, 2). It has since been shown that this property is due to the lactogenic hormone,[2] while the growth hormone shows the opposite action of decreasing pancreatic insulin (3). The English workers have further demonstrated that estrogens increase the insulin content of rat pancreas (5, 6), an effect which has since been interpreted as mediated by the pituitary (7).

Concerning an interrelationship between the thyroid and the pancreas, various claims have been made (8, 9). It was therefore decided to study the effect of thyroxin on pancreatic insulin content. The thyroid hormone is known to show variable effects dependent upon the dose administered. Thus at low doses it synergizes with growth hormone while at high doses it causes loss of weight. To be able to observe its effect at physiological dose levels, it was decided to use hypophysectomized rats, administering a dose which would bring their oxygen consumption back to that of normal rats.

Various groups of hypophysectomized rats of both sexes were treated daily for periods of 10 to 21 days with approximately 0.01 mg. thyroxin per 100 gm. of rat weight. As the data in table 1 show, the oxygen consumption of these rats was approximately normal, and no weight loss occurred under these conditions. In every case the pancreatic insulin content of the treated rats was about half that of their controls.[3] In one experiment normal rats were used which were rendered distinctly hyperthyroid by administration of twice the dose of thyroxin given to hypophysectomized rats. In these normal rats thyroxin produced no decrease but actually an increase in pancreatic insulin.

DISCUSSION

Thyroxin at physiological doses has been demonstrated to cause a decrease in the pancreatic insulin content of hypophysectomized rats. Administration of thy-

Received for publication December 29, 1941.

[1] Aided by grants from the Board of Research of the University of California and the Rockefeller Foundation, New York City, and Parke, Davis & Company, Detroit, Michigan. We wish to acknowledge assistance from the Work Projects Administration, Project No. OP-65-1-08, Unit A-5.

[2] Funk et al. (4) have recently claimed lactogenic hormone to be "diabetogenic," on the basis of striking decreases in pancreatic insulin which they claim to have obtained by the administration of this hormone. All attempts on our part to duplicate such findings were unsuccessful; on the contrary, pure lactogenic hormone was found to increase pancreatic insulin to double that of the controls, even under the conditions used by these authors (0.25 mg. daily of pure lactogenic hormone for 8 days in normal male rats). Also blood sugar determination did not indicate a diabetogenic action of this hormone (98 mg.% in injected and control rats).

[3] For method of assay of pancreatic insulin cf. reference 3, 7.

roxin to normal rats appears to have the opposite effect. Similar differences between the physiological rôle of the thyroid and the hyperthyroid state are known to occur in respect to nitrogen balance and in other phases of metabolism. Thus it would appear that the hypophysectomized (or thyroidectomized) animal is a better test object than the normal when the physiological action of the thyroid is under investigation.

The significance of the observed decrease in pancreatic insulin content is not easily interpreted. In the case of the pituitary hormones there are some indications

TABLE 1. EFFECT OF THYROXIN ON PANCREATIC INSULIN CONTENT

Exp. No.	Daily dose per rat	Injection period	Rats			O_2 consumption	Body wt.[4]	Insulin content	
			Type	Age[1]	Post-op.[1]			u/100 gm. rat	Change
	mg.	*days*		*days*	*days*	*l/m²/day*	*gm.*		*%*
1	0.0075	10	♀	43– 45	17–19	183	74	0.25	−56
	Controls			43– 45	17–19	129	70	0.56	
2	0.0075	21	♀	54– 56	28–30		81	0.44	−52
	Controls			54– 56	28–30		74	0.92	
3	0.015	10	♂	75–100	24–28	168	134	0.53	−33
	Controls			75–100	24–28	126	138	0.79	
4	0.015	17	♂	80–110	30–45	166	138	0.48	−47
	Controls			80–110	30–45	124	139	0.90	
5	0.04–0.05[2]	21	Norm. ♂	76– 77		268[3]	264	0.33	+38
	Controls			76– 77		214	280	0.24	

[1] On day of autopsy.
[2] 0.04 mg. for 8 days, then 0.05 mg. until end.
[3] On twelfth day of injection period.
[4] At onset of 24-hour fast, preceding autopsy.

that increased insulin content may be associated with decreased secretion and vice versa.[4] Similarly thyroxin may cause a decrease in pancreatic insulin through stimulation of its secretion. The finding by Zunz and La Barre and others of increased blood insulin after thyroid stimulation favors this assumption (8, 9).

SUMMARY

Thyroxin at physiological levels has been shown to decrease the pancreatic insulin content of hypophysectomized rats.

REFERENCES

1. MARKS, H. P., AND F. G. YOUNG: *Lancet* 493. 1940.
2. SOONG, N. Y.: *Chinese J. Physiol.* 15: 335. 1940.
3. FRAENKEL-CONRAT, H. L., V. V. HERRING, M. E. SIMPSON AND H. M. EVANS: *Am. J. Physiol.* In press. 1942.
4. FUNK, C., T. M. CHAMELIN, H. WAGREICH AND B. HARROW: *Science* 94: 260. 1941.
5. GRIFFITHS, M., AND F. G. YOUNG: *Nature* 146: 266. 1940.
6. GRIFFITHS, M., AND F. G. YOUNG: *Nature* 147: 359. 1941.
7. FRAENKEL-CONRAT, H. L., V. V. HERRING, M. E. SIMPSON AND H. M. EVANS: *Proc. Soc. Exp. Biol. & Med.* 48: 333. 1941.
8. ZUNZ, E., AND J. LA BARRE: *Arch. internat. de Physiol.* 42: 1935.
9. KOTCHNEFF, N. P., AND E. S. LONDON: *Fisiol.* 22: 372. 1937.
10. BEST, C. H., J. CAMPBELL AND R. E. HAIST: *J. Physiol.* 97: 200. 1939.

[4] This must not be confused with the finding of Best et al. (10) that in the dog exhaustion of the islet tissue and concomitant diabetes is accompanied by extremely low pancreatic insulin content.

RADIOACTIVE IODINE AS AN INDICATOR OF THE METABOLISM OF IODINE

IV. THE DISTRIBUTION OF LABELED THYROXINE AND DIIODOTYROSINE IN LIVER, MUSCLE AND SMALL INTESTINE[1]

I. PERLMAN,[2] M. E. MORTON AND I. L. CHAIKOFF[3]

From the Division of Physiology of the Medical School, University of California

BERKELEY, CALIFORNIA

BY THE USE OF radioiodine it has been shown that circulating iodide rapidly appears in the thyroid gland as organic compounds that can be separated into diiodotyrosine-like and thyroxine-like fractions (1, 2). A larger portion of the gland's radioiodine was always present as diiodotyrosine than as thyroxine. In the plasma these labeled organic iodine compounds were present as early as two hours after the introduction of radioiodine. At this early interval the level of organically-bound radioiodine was low in plasma, and the diiodotyrosine fraction predominates. At later intervals the amount of organically-bound radioiodine increased, and the thyroxine-like fraction in the plasma then far exceeded the diiodotyrosine-like fraction, even though the major fraction of the gland's labeled iodine was still present as diiodotyrosine.

The gland made hyperactive by the injection of thyrotropic hormone was characterized by a greater uptake of circulating radioiodine than the normal gland (2). In addition a larger portion of the thyroid's radioiodine is present as thyroxine in the hyperactive than in the normal gland. At the two-hour interval 10 per cent of the normal gland's radioiodine was found as thyroxine, whereas in the hyperactive gland 20 per cent of its radioiodine was so held. Interestingly enough, at the latest interval investigated, namely 26 hours after the administration of the radioiodine, 80 per cent of the plasma's radioiodine was contained as thyroxine. In other words, circulating radioiodide present at any particular time will be largely converted or replaced by thyroxine-like radioiodine in the course of 24 hours.

The above studies have dealt with the withdrawal of injected iodine by the thyroid gland, its appearance in the gland as organic compounds and the subsequent appearance of these compounds in plasma. The further course of these thyroxine-like and diiodotyrosine-like compounds obviously involves the peripheral tissues.[4] Hence the manner in which administered radioiodine makes its appearance in muscle, liver and small intestine has been made the subject of the present investigation.

EXPERIMENTAL PROCEDURE

In the first series of experiments 12 male guinea pigs averaging 330 gm. (310–350 gm.) were used. They were divided into 2 groups of 6 animals, one group of which re-

Received for publication December 9, 1941.

[1] Aided by a grant from the Committee on Research in Endocrinology of the National Research Council.

[2] Upjohn Research Fellow.

[3] Fellow of the John Simon Guggenheim Memorial Foundation.

[4] In these studies, the term 'peripheral tissue' refers to any tissue other than thyroid and plasma.

ceived subcutaneously once daily for 4 days 1 mg. of a thyrotropic hormone preparation,[5] and the other served as control. On the fifth day all 12 guinea pigs received intraperitoneally 1 cc. of a solution containing tracer amounts[6] of radioiodine. After 2 hours, 3 thyrotropic-hormone-treated and 3 control animals were killed by a blow on the neck; the other 6 animals were sacrificed at the 26-hour interval. The small intestine was rapidly removed, washed with isotonic saline, weighed and minced, and approximately 5 gm. samples taken for analysis. Liver and gastrocnemius muscle were weighed, chopped and sampled. In some cases somewhat less than 5 gm. of muscle was obtained from both gastrocnemii; in such cases a small amount of muscle was taken from the thigh.

All samples were hydrolyzed on the steam bath with 90 cc. of 2N NaOH for 8 hours. After cooling, they were made up to volume in 100 cc. volumetric flasks. Five or 10 cc. were ashed directly in a manner previously described in order to obtain an independent measurement of the total labeled iodine content present in the tissue (3). The remaining 90 or 95 cc. were separated into 3 fractions according to the method fully described elsewhere (1). The two butyl alcohol extractions used to remove thyroxine were made with a 30 cc. and a 20 cc. portion, after which the combined butyl alcohol layer was washed with two 20 cc. portions of 20 per cent NaOH. In all other respects the method was the same as that previously described (1, 2).

The second series of experiments was carried out on 18 female guinea pigs weighing about 350 gm. Nine of the animals received over a 6-day period 5 injections of 1 mg. of the thyrotropic hormone preparation. On the seventh day all 18 animals received radioiodine intraperitoneally. Three hormone-treated animals and 3 control animals were killed at 2, 26, and 96 hours after I* administration. The 3 hormone-treated ones that were sacrificed at the 96-hour interval had been injected with an additional 1.0 mg. of the thyrotropic hormone preparation 48 hours after they received the radioiodine. Small intestines alone were taken from this series of 18 guinea pigs. The sampling and analyses were carried out as described above.

RESULTS

It should be recalled that tracer amounts of radioiodide were administered. The amount of iodide introduced into the animal in this manner is very minute and in all probability does not affect the concentration of the iodide already present.

Iodine in all its forms is not immediately labeled merely by introducing radioactive *iodide* into the circulation. From the way in which it was prepared from the tellurium target after removal from the cyclotron, it is known that radioiodine was injected as iodide. Hence, its most immediate action after its introduction into the bloodstream is to label plasma *iodide* as well as tissue iodide that comes into rapid equilibrium with plasma.

After inorganic circulating iodine is labeled in this manner, radioactive measurements may fulfill a twofold purpose. First, if the radioactivity of the inorganic iodine

[5] The thyrotropic preparation used in this investigation was furnished by Dr. Q. Bartz of Parke, Davis and Company, Detroit, Michigan to whom our thanks are due. This fraction assayed 4 guinea-pig units per mg. The unit is defined as the total dose in mg. injected subcutaneously once daily for 4 days into six 180 and 200 mg. guinea pigs, producing on the 5th day minimal but definite hyperplasia of the thyroid in all six animals.

[6] The method by which tracer amounts of radioiodine are prepared has been simplified over that previously described (3). The tellurium, after removal from the target, is placed in a distilling flask and cautiously heated with chromium trioxide and H_2SO_4 until solution is complete. An excess of oxalic acid is added and, after the reaction has stopped, 25 cc. of 1:1 H_2SO_4 is poured in through a side arm. Distillation is then carried out into an alkaline solution containing a small amount of bisulfite. After neutralization, the solution is ready for use.

fraction of a tissue is measured as a function of time, the manner in which inorganic circulating iodine enters and leaves a tissue may be determined. Second, the entrance of iodide into organic compounds may be observed by measuring the radioactivity of the organic fractions.

One difficulty presents itself in the interpretation of results based solely on radioactive measurements. When comparing animals, particularly under different conditions, we may find different levels of circulating iodine. The same quantity of radioactivity may then serve to label different absolute amounts of iodide. Little therefore can be said about the absolute quantity of iodine represented by the transport or conversion of a specified amount of radioactivity. In each animal, however, the fate of a particular portion of I* represents the fate of a certain fraction of the initially labeled total circulating iodide. In other words, the degree to which the entire initially labeled fraction enters into organic compounds may be compared in normal and thyrotropic-hormone-treated animals by means of the radioactive measurements, but the absolute amounts of iodide involved in such conversions can be compared only when the actual amounts of initially labeled iodide are known.

TABLE 1. RADIOIODINE DISTRIBUTION IN LIVER, MUSCLE, AND SMALL INTESTINE

Tissue	Time after I* administration (1)	Normal Guinea Pigs: guinea pig No. (2)	Normal Guinea Pigs: % of administered I* per gm. tissue×10 (3)	Normal Guinea Pigs: % of tissue I* found as: Thyroxine (4)	Diiodotyrosine (5)	Inorganic (6)	Total (7)	Thyrotropic-Hormone-Treated Guinea Pigs: guinea pig No. (8)	% of administered I* per gm. tissue×10 (9)	% of tissue I* found as: Thyroxine (10)	Diiodotyrosine (11)	Inorganic (12)	Total (13)
Liver	2	57	0.486	3.1	10.3	86.5	100	54	0.492	18.1	18.7	63.5	100
	2	58	0.426	1.01	1.12	97.5	100	55	0.392	16.0	1.93	81.2	99
	2	59	0.460	0.94	4.23	99.5	105	56	0.368	21.0	4.28	77.6	103
	26	63	0.0790	17.9	11.3	70.0	99	60	0.962	61.0	18.6	20.4	100
	26	64	0.0687	20.8	11.7	67.5	100	61	0.920	60.7	18.0	21.3	100
	26	65	0.0598	18.6	13.8	67.6	100	62	1.07	59.3	19.6	21.1	100
Muscle	2	57	0.294	1.5	7.90	90.5	100	54	0.246	6.04	6.39	87.5	100
	2	58	0.283	2.77	2.68	94.5	100	55	0.205	5.42	3.88	86.0	95
	2	59	0.331	2.37	8.06	89.5	100	56	0.188	12.8	2.19	85.0	100
	26	63	0.0527	11.2	7.74	81.4	100	60	0.251	55.6	10.4	34.0	100
	26	64	0.0275	37.8	6.92	55.4	100	61	0.154	61.6	13.0	24.4	99
	26	65	0.0215	35.6	7.76	47.5	91	62	0.0716	68.8	5.86	26.2	101
Small Intestine	2	57	0.932	0.99	5.61	97.0	104	54	1.88	1.90	6.64	87.7	96
	2	58	2.28	0.825	7.22	92.0	100	55	2.44	1.27	7.84	87.6	97
	26	63	0.139	3.94	7.77	87.0	99	60	0.354	73.0	5.16	23.9	102
	26	64	0.0741	14.5	5.42	80.0	100	61	0.216	79.2	4.10	20.3	104

The data obtained from liver, muscle and small intestine are shown in table 1. *Columns* 3 and 9 show the percentage of the administered radioiodine recovered per gram of tissue in normal and hyperactive guinea pigs, respectively. These values were obtained by direct determination of the total radioactivity in an aliquot of the tissue hydrolysate. Obviously the figures shown in these columns give only a general picture of iodine movement in and out of a tissue and throw no light on the forms in which iodine appears. For this reason the differences in radioiodine levels observed between hyperactive and normal animals as a function of time are best discussed along with the iodine fractions which make up the total radioiodine content. In *columns* 4 to 6 and 10 to 12 is shown the percentage of the total radioiodine content which is found in the three fractions: thyroxine, diiodotyrosine and iodide. *Columns* 7 and 13 represent the sum of I* recovered by the addition of the 3 separate fractions; this is expressed as a percentage of the value shown in *columns* 3 and 9. *Columns* 7 and 13 thus show the reliability of the recovery of I* after fractionation.

Relation betweeen Organic I and Total I**

Normal animals. Since, at the time of labeling, all radioactivity is in the inorganic fraction, the difference between total I* and inorganic I* at any time indicates the degree to which organic binding has proceeded. In the normal guinea pig tissues, it is seen from table 1, the total radioiodine content is high at 2 hours (as compared with later intervals) and almost all of it is still in inorganic form. Values in *column* 6 show that in the liver of the normal guinea pig 86.5 to 99.5 per cent of its radioiodine is in the inorganic form. In muscle and small intestine similar values were obtained, namely 89.5 to 94.5 per cent and 92 to 97 per cent, respectively. These results fall within the same range as those previously reported for plasma (2). In normal guinea pigs, 87 to 99 per cent of the plasma's I* was found as iodide at the 2-hour interval. The results show that in 2 hours that part of the cycle,[7] in which injected iodide enters the circulation, is converted to organic iodine and then appears in these organic forms in the circulation and peripheral tissues, does not occur to any appreciable degree in the normal guinea pig.

Hyperactive animals. The animals treated with thyrotropic hormone present a somewhat different picture. Two hours after the administration of the radioiodine, the total I* contents of liver, muscle and small intestine of the treated animals were similar to those in corresponding tissues of the normal animal. However, a larger proportion of each tissue's I* was organically bound in the hormone-treated than in the normal animal. The most striking difference was observed in the case of the liver (*column* 12), in which 63.5 to 81 per cent of the I* was found in the inorganic fraction; in other words, 20 to 35 per cent was already organically bound at the 2-hour interval. In small intestine and muscle, differences between the normal and hyperactive animals were less marked. This corresponds to the picture previously reported for plasma; more of the plasma's I* was in the organic form in the thyrotropic-hormone-treated animal than in the normal guinea pig. In 2 hours, over 40 per cent of plasma I* was organically bound in the treated animals. The above differences indicate that in the hyperactive animal a larger proportion of the initially labeled iodide is constantly being converted to organic iodine and is making its appearance in organic form in plasma and peripheral tissues.

Turnover of inorganic iodine. Previous studies on normal rats and rabbits showed that soon after the injection of radioiodide tissues other than thyroid showed a maximum retention of total radioactivity, which dropped off rapidly with time (3). The parallelism between tissues and blood was also noted. These findings made it seem likely that the movement of iodine into and out of the tissues was largely a diffusion of iodide between plasma and tissue fluid, a process presumably governed by the level of plasma iodide. This view was previously presented by Wallace and Brodie (4) and was based on the distribution of massive doses of iodide between tissues and plasma. In their studies iodide behaved like chloride and presumably was free to diffuse between plasma and extracellular tissue fluid. However, since it is likely that the organism has a limited capacity for converting iodide to organic iodine, the administration of iodine far in excess of the physiological capacity would be expected to obscure any changes that might occur in the small quantity actually utilized.

In the present study, however, by the use of tracer amounts of radioiodide it is shown that tissue iodide is constantly turning over in more than one respect. As in the above studies (3, 4), it is found that the inorganic iodine does rapidly diffuse into and out of tissues, as evidenced by the rapid appearance of radioactivity soon after

[7] Cycle refers to the sequence of reactions in which inorganic iodine is converted to organic forms and back to iodide.

the administration of radioiodine and by the fact that this radioactivity is largely in the inorganic fraction. On the other hand, as time goes on, the inorganic radioiodine disappears and is replaced by organically bound radioiodine. The possibility that organification of iodine may take place elsewhere than in the thyroid gland must not be overlooked.

In the tissues of normal guinea pigs, the marked drop in radioiodine content between 2 and 26 hours is seen from *column* 3 of table 1. The liver shows a decrease in radioactivity from about 0.45 to 0.07, a 7-fold change, while muscle and small intestine values decrease in comparable manner. *Column* 6 shows that in the liver, small intestine, and in 2 of 3 cases in muscle, radioiodine after 26 hours is still mostly inorganic. Almost 70 per cent of liver's radioiodine is present as iodide, in the small intestine over 80 per cent is in this form, while for muscle the values vary from 48 to 81 per cent. It is therefore reasonable to suppose that the marked drop with time in the tissues of normal guinea pigs represents an outward diffusion of radioiodide as blood radioiodide becomes depleted through withdrawal by the thyroid, excretion.

In the thyrotropic-hormone-treated animals, total I* content of small intestine and muscle did not suffer nearly as great a decrease between 2 and 26 hours as did these tissues in the normal animal, while the livers actually showed an increase (*column* 9). The radioiodine contents of muscle at 2 and 26 hours were, respectively, 0.19–0.25 and 0.07–0.25. In the experiment shown in table 1, the content in the small intestine dropped considerably, but in another experiment (to be described below: table 2, *column* 9), the decrease in radioactivity was similar to that of muscle. The liver (table 1) increased almost twofold in radioiodine content between 2 and 26 hours. In general, it may be stated that the hormone-treated animals maintain their initial high level of tissue radioiodine much better than do the normal animals. However, the relatively high levels at 26 hours are not due to retention of the inorganic iodine which was present at 2 hours. *Column* 12 in table 1 shows that only about 20 to 25 per cent or each tissue's iodine is inorganic at 26 hours. Apparently inorganic radioiodine has left these tissues, as in the normal animals, but it has been replaced by radioiodine recently synthesized into organic compounds. In other words, the radioiodide present at a given time in the tissues of guinea pigs treated with thyrotropic hormone will be largely replaced by organic radioiodine within 24 hours. In contrast, the tissue radioiodide of the normal animal will still be present largely as radioiodide after the same interval.

Thyroxine and Diiodotyrosine

Distribution up to 26 hours. The organically bound I* discussed above represented the difference between total I* and iodide I* and was separated into a thyroxine-like fraction and diiodotyrosine-like fraction. The fraction most affected by time and thyroid activity was thyroxine; the response in diiodotyrosine fraction was more irregular and showed no definite trend with time and thyroid activity.

Again it should be recalled that the inorganic circulating iodine is the first to be labeled with injected I*. The appearance of radioactivity in diiodotyrosine and thyroxine fractions therefore indicates synthesis from inorganic iodine.

In previous studies (1, 2) it was found that the greatest percentage of the thyroid's I* always appeared in the diiodotyrosine fraction. Such was not the case in the plasma. At the end of one day, the percentage of plasma's I*[8] found as thyroxine was greater than that found as diiodotyrosine. This was true for normal animals as well as for those whose thyroid glands were made hyperactive by treatments with thyrotropic

[8] I* and radioiodine are used interchangeably.

hormone. In the present study the distribution of radioiodine between these two organic fractions in liver, muscle and small intestine followed the same general outline as in plasma rather than in the thyroid gland.

In normal guinea pigs, only small amounts of organic radioiodine were present in the tissues at 2 hours, and most of this was in the diiodotyrosine fraction (tables 1 and 2, *columns* 4 and 5). The radio-diiodotyrosine expressed as percentage of the total radioiodine varied from 1.1 to 10.3, with most of the values lying between 5 and 10. Expressed in the same manner, the thyroxine fraction showed values from 0.8 to 3.1.

The thyrotropic-hormone-treated animals showed a somewhat more rapid conversion of their iodide to organic iodine than the normal, the difference in the two types of animals occurring principally in the thyroxine fraction. At 2 hours the percentage of each tissue's I* found as diiodotyrosine did not differ in normal and hyper-

TABLE 2. RADIOIODINE DISTRIBUTION IN SMALL INTESTINE

Time after I* administration	Normal Guinea Pigs						Thyrotropic-Hormone-Treated Guinea Pigs					
	guinea pig No.	% of administered I* per gm. tissue×10	% of tissue I* found as				guinea pig No.	% of administered I* per gm. tissue×10	% of tissue I* found as			
			Thyroxine	Diiodotyrosine	Inorganic	Total			Thyroxine	Diiodotyrosine	Inorganic	Total
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
2	77	0.981	2.41	9.12	91.4	103	68	0.754	5.89	9.40	88.0	103
2	78	1.40	1.13	9.60	90.0	101	69	0.301	11.2	14.9	77.0	103
2	79	1.28	1.44	10.2	87.6	99	70	0.818	6.47	10.2	78.9	96
25	80	0.208	29.7	5.30	65.0	100	71	0.606	62.3	6.23	32.9	101
25	81	0.261	14.2	7.17	79.5	101	72	0.469	61.7	5.02	33.3	100
25	82	0.178	16.6	6.50	78.4	102	73	0.496	60.2	5.94	32.3	98
97	83	0.0444	55.7	11.9	27.7	95	74	0.124	64.2	13.8	25.4	103
97	84	0.0248	56.2	10.6	28.2	95	75	0.0763	60.0	12.2	24.8	97
97	85	0.0423	48.8	13.4	36.2	98	76	0.0905	61.2	14.0	25.9	101

active animals. At this same time-interval, however, thyroxine values in hyperactive animals were considerably higher than in their controls, especially as regards the liver, in which 21 per cent of the total radioiodine was already in the form of thyroxine.

Between 2 and 26 hours there was a great increase in the relative quantity of radiothyroxine in the tissues of both normal and hyperactive animals. Although considerable variations were encountered, between 15 and 30 per cent of the radioiodine was in the form of thyroxine at 26 hours in most of the normal guinea pigs, as compared with 1 to 2.5 per cent at the earlier hour. Hyperactive animals thus showed a much more complete conversion of initially labeled iodide to thyroxine. At 26 hours, 59 to 61 per cent of the liver's I* was in the form of thyroxine, 60 to 79 per cent in the small intestine (tables 1 and 2), and 56 to 69 per cent in muscle.

In contrast to thyroxine I*, the amount of which progressively increases in the plasma and peripheral tissues, diiodotyrosine I* was low at both time intervals. If the conversion of iodide to thyroxine and diiodotyrosine takes place *only in* the thyroid gland, then it follows that there is a preferential output of thyroxine into plasma and into peripheral tissues, since in the gland the amount of newly formed radiodiiodotyrosine exceeds that of newly formed radiothyroxine. If on the other hand, diiodotyrosine can be formed in the peripheral tissues, then it is likely that the thyroid removed diiodotyrosine from the plasma. This follows from the finding that newly formed diiodotyrosine does not accumulate in the peripheral tissues. Of course, diiodotyrosine could be prevented from accumulating in these tissues through other mechanisms, such as breakdown to yield iodide or further synthesis to thyroxine. The likelihood that the latter proceeds to any appreciable extent in tissues other than the thyroid is not very great.

Distribution at later intervals. The latest interval studied was 96 hours. Such extended observations were carried out only in the small intestine. Figure 1 shows the distribution of I* between thyroxine and inorganic fractions in the small intestine at 2, 26 and 96 hours. In the hormone-treated animals the percentage of total I* found as iodide and thyroxine do not change appreciably between 26 and 96 hours; in the normal or control animals the thyroxine I* continues to increase and the iodide I* to decrease between these two time intervals. The more rapid attainment of a steady condition in the small intestine of animals with thyroids made hyperactive may be due

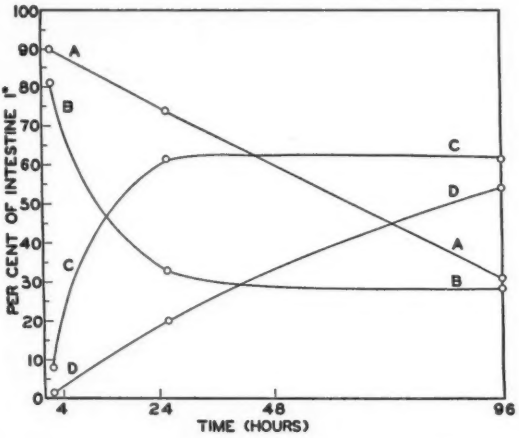


Fig. 1. Distribution of small intestine's I* between thyroxine and inorganic fractions. *A*, Inorganic I*, normal guinea pig; *B*, inorganic I*, hyperactive guinea pig; *C*, thyroxine I*, hyperactive guinea pig; *D*, thyroxine I*, normal guinea pig. Each point is the average of 3 values shown in table 2.

to a more rapid removal of the initially labeled circulating iodide by the thyroid gland, a more rapid conversion of this to thyroxine, and a more rapid release of thyroxine by the gland into plasma and peripheral tissues.

It should be noted here again that the injection of a tracer dose of I* does not increase the actual content of iodine in the body; hence the transport of I* in its various forms as measured here portrays a phase of endogenous iodine cycle. The decrease in the inorganic I* fraction can only mean that the specific activity[9] of this fraction is decreasing, since it may reasonably be assumed that the total inorganic iodine remains unchanged during the period of observation. The fall in specific activity of the iodide I* is probably the result of two processes: *a*), excretion of labeled iodide, taking place concurrently with intake of unlabeled iodide; and *b*), conversion of iodide I* to organic forms and the regeneration of iodide at the expense of these organic forms. At the very early intervals after the injection of I*, the organic iodine is unlabeled and hence the iodide liberated is unlabeled. The addition of this unlabeled iodide to the system accounts for the rapid drop in specific activities at the early intervals. At somewhat later intervals, the fractions are partially labeled; their breakdown now contributes a certain amount of I*. It can be readily seen that as this process proceeds the drop in specific activity becomes less and less. In a system in which the intake and excretion of iodine are negligible as compared to the amount going through the cycle a time will be reached when the percentage of I* in each fraction will no

[9] Specific activity is defined as I*/I, i.e., the radioactivity associated with unit quantity of iodine.

longer change. When iodine throughout the body is equally labeled, i.e. when unit amounts of iodine in any part of the body will be associated with the same amount of I*, conversion of iodine from one form to another can not change the specific activity of either form. Although for practical purposes we may speak of complete labeling, it should be noted that complete labeling is a limit that is only approached asymptotically.

In figure 1 the distribution of the small intestine's I* between iodide and thyroxine is shown. The results obtained here may be regarded as typical for other peripheral tissues.[10] In the hyperactive animal, a sharp drop in the iodide I* was observed between the first and second intervals; this was accompanied by a sharp rise in the thyroxine-like I*. No appreciable change in the distribution of this tissue's I* between iodide and thyroxine occurred after 25 hours. The values found for the small intestine's I* as iodide were 30 per cent at both 25 hours and 96 hours. The values for thyroxine were 60 per cent at both time intervals. This means that all iodine fractions are about equally labeled in about 24 hours. A longer time is necessary for complete labeling in the normal than in the hormone-treated animal. From the data shown in table 2 and figure 1 it is not possible to state, however, whether *complete* labeling has been attained even in 96 hours.

As pointed out above, all iodine fractions possess the same specific activity when equilibrium is attained, i.e. when labeling is complete. At this time the distribution of I* among its fractions should be in proportion to the distribution of total iodine. Hence when equilibrium is reached, the distribution of I* should be a measure of the relative amounts of iodide, thyroxine and diiodotyrosine. According to this interpretation, the total iodine of the small intestine of the hyperactive animal is distributed among iodide, thyroxine and diiodotyrosine in the ratio of 30:60:10, respectively.

SUMMARY

The deposition of radioiodide, radiothyroxine and radiodiiodotyrosine was measured in liver, muscle and small intestine with radioactive iodine as indicator.

The character of the uptake and loss of total radioiodine by these 3 tissues resembled that of plasma.

The distribution of each tissue's I* among the 3 fractions, iodide, thyroxine and diiodotyrosine, changed with time but at each time interval the distribution resembled that observed in the plasma.

Almost all of the initially labeled iodide in these tissues is replaced by labeled organic iodine in 26 hours by the animal whose thyroid glands had been made hyperactive by injections of the thyrotropic hormone. In the normal animal more than half of the initially labeled iodide is still in the inorganic form at 25 hours.

The percentage of each tissue's I* found as thyroxine increased with time.

The diiodotyrosine fraction as a percentage of each tissue's total I* remained low and relatively uninfluenced by time.

The significance of these findings in relation to the iodine cycle is discussed.

REFERENCES

1. Perlman, I., M. E. Morton and I. L. Chaikoff: *J. Biol. Chem.* 139: 449. 1941.
2. Morton, M. E., I. Perlman and I. L. Chaikoff: *J. Biol. Chem.* 140: 603. 1941.
3. Perlman, I., I. L. Chaikoff and M. E. Morton: *J. Biol. Chem.* 139: 433. 1941.
4. Wallace, G. B., and B. B. Brodie: *J. Pharmacol. & Exper. Therap.* 65: 214. 1939.
5. Elmer, A. W.: Iodine Metabolism and Thyroid Function. London, 1938. P. 269.

[10] It should not be inferred from the above statements that no differences exist in the distribution of I* among thyroxine, diiodotyrosine and iodide in small intestine, liver and muscle. It has been suggested that some tissues other than thyroid (e.g. alimentary tract) may iodinate tyrosine (5).

RADIOACTIVE IODINE AS AN INDICATOR OF THE METABOLISM OF IODINE

V. THE EFFECTS OF HYPOPHYSECTOMY ON THE DISTRIBUTION OF LABELED THYROXINE AND DIIODOTYROSINE IN THYROID GLAND AND PLASMA[1]

M. E. MORTON, I. PERLMAN,[2] EVELYN ANDERSON AND I. L. CHAIKOFF[3]

From the Division of Physiology of the Medical School and the Institute of Experimental Biology, University of California

BERKELEY, CALIFORNIA

THE EFFECTS OF hypophysectomy in depressing thyroid gland activity have been known for some time. Very little, however, has appeared on the iodine metabolism of the hypophysectomized animal, and a description of the thyroxine and diiodotyrosine contents of the thyroid gland and other tissues of the hypophysectomized animal is lacking. The information available on the hormone production of the thyroid gland of the hypophysectomized animal has been inferred largely from two sources, *a*), B.M.R. measurements, and *b*), histological examination of the gland.

In previous reports, the conversion of I* to labeled thyroxine and diiodotyrosine in the tissues of the normal animal was compared with that in the animal whose thyroid gland had been made hyperactive by the administration of thyrotropic hormone. The present paper deals with the metabolism of I* and its thyroxine-like and diiodotyrosine-like fractions in tissues of animals in which the thyroid gland had been depressed by total hypophysectomy.

EXPERIMENTAL PROCEDURE

Two-month-old rats were hypophysectomized by the parapharyngeal route. At the same time normal rats of the same age were set aside to be used as controls. Completeness of hypophysectomy was evidenced by loss of body weight, atrophy of thyroids, adrenals and testes, and by absence of pituitary fragments at postmortem examination. All animals were kept on a stock diet throughout the course of the experiment. Nine or 10 days after hypophysectomy each of the animals was injected intraperitoneally with 1 cc. of a solution containing tracer amounts of radioiodide. The preparation of radioiodine for use in such experiments has already been described (2, 3). At different time intervals, groups of 3 hypophysectomized rats and 3 normal rats were anesthetized with nembutal and blood was removed by heart puncture and centrifuged. Thyroids, adrenals and testes were also removed and weighed. Thyroids were pooled from the 3 rats in each group, as were also 2 cc. samples of plasma from each rat. The 6 cc. of plasma so obtained was hydrolyzed with 50 cc. of 2N NaOH

Received for publication December 9, 1941.

[1] Aided by a grant from the Committee on Research in Endocrinology of the National Research Council.

[2] Upjohn Research Fellow.

[3] Fellow of the John Simon Guggenheim Memorial Foundation.

for 8 hours on the steam bath. The thyroids were similarly treated with 20 cc. of 2N NaOH after adding 200 mg. of desiccated thyroid. The fractionation of the thyroid and plasma hydrolysates into thyroxine, diiodotyrosine and inorganic iodine has been described elsewhere (4, 1).

Two series of experiments were carried out; they differed only in the time between the administration of radioiodine and the sampling of tissues. In the first series, groups of rats were autopsied at 2 and 26 hours, while in the second series the time intervals were 1, 4, 12, 36 and 96 hours.

RESULTS

The 'tracer' dose of iodine was employed in this study. As noted elsewhere, the amount of iodine so injected does not alter the iodine content of the animal. The significance of this type of labeling procedure has been dealt with at length in the preceding paper (2) and applies equally well to the data recorded below.

Uptake of total I in thyroid gland* (*fig. 1*). The comparison between normal and hypophysectomized rats is striking. At 4 hours the thyroid of the normal animal had taken up 60 per cent of the administered I*; the thyroid gland of the hypophysectomized rat contained less than 5 per cent of the total I* it received by injection. The results obtained by Leblond, Sue, and Chamorro (5) point in the same direction.[4]

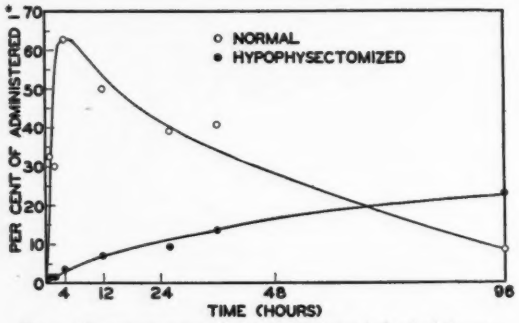


Fig. 1. Uptake of total I* by whole thyroid gland of the rat.

Figure 1 shows that a progressive decrease in the I* content of the thyroid gland of the normal animal occurs after the 4-hour interval. In the hypophysectomized animal, however, the I* content of the thyroid gland continued to rise slowly throughout the entire period, namely 96 hours.

I content of plasma* (fig. 2). Differences between hypophysectomized and normal rats are again apparent. High levels of I* were observed in both types of animals at the one-hour interval. The I* left the blood more rapidly in the normal than in the hypophysectomized rats. Thus at both the 1 and 4-hour intervals, approximately the same high levels were found in the hypophysectomized rats, whereas in the normal animals the I* content at 4 hours was about one-third of that found at one hour. The content of I* in the blood of the hypophysectomized rat continued to drop from 4 to 96 hours. In the normal, the contents at 12 and 36 hours were practically the same, but at 96 hours an increase above the latter values had occurred. In the normal ani-

[4] The difference in the uptake of I* observed by these workers between the thyroid glands of normal rats and the thyroid glands of rats 9 days after hypophysectomy was not as great as that found here. It should be noted, however, that tracer doses of I* were employed in the present investigation whereas the sample of labeled iodine used by Leblond et al. contained considerable quantities of iodine.

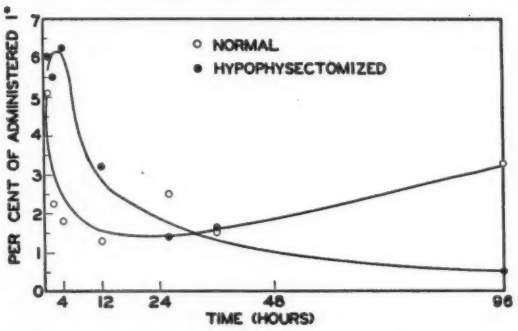


Fig. 2. Uptake of total I* by plasma of the rat. The ordinates represent the percentage of the administered I* found in the entire plasma of the rat. Entire plasma was estimated as 5 per cent of the body weight.

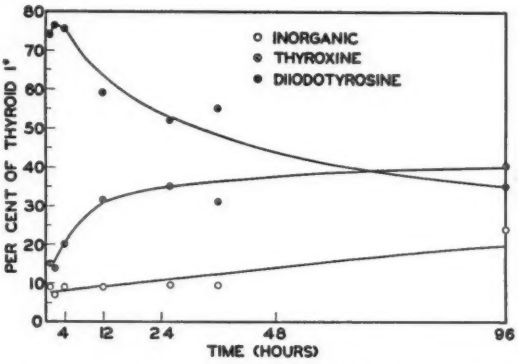


Fig. 3. Distribution of thyroid's I* among inorganic, diiodotyrosine and thyroxine fractions in normal rat.

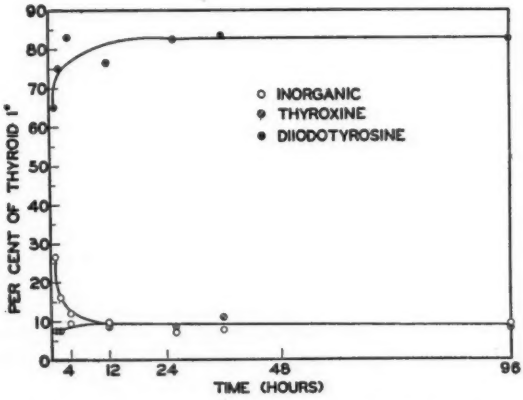


Fig. 4. Distribution of thyroid's I* among inorganic, diiodotyrosine and thyroxine fractions of the hypophysectomized rat.

mals, the rapid drop in plasma I* occurring up to 4 hours was coincidental with the rapid uptake of I* by the thyroid. Similarly, the slow rise in plasma I* from 12 to 96 hours took place at the same time that the thyroids were releasing I*. At 96 hours the percentage of the injected labeled iodine found in the blood of the normal was

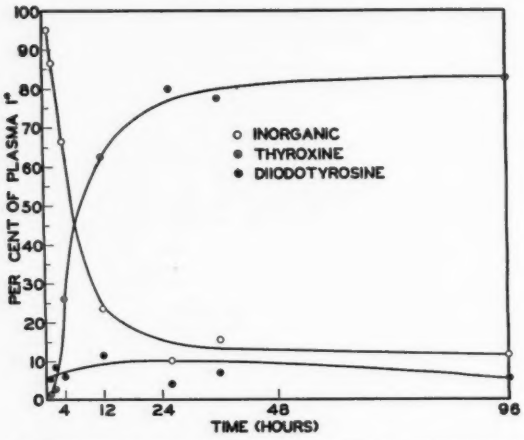


Fig. 5. Distribution of plasma's I* among inorganic, diiodotyrosine and thyroxine fractions of the normal rat.

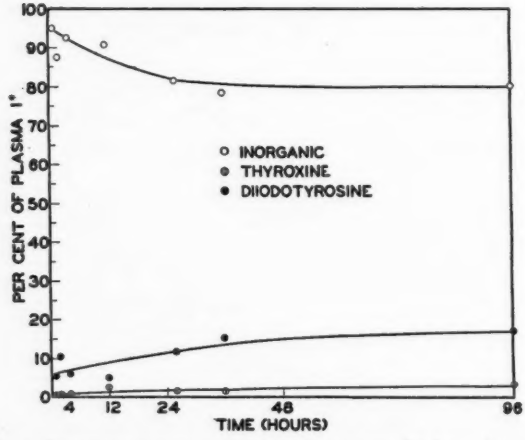


Fig. 6. Distribution of plasma's I* among inorganic, diiodotyrosine and thyroxine fractions of the hypophysectomized rat.

approximately 6 times that in the blood of the hypophysectomized animal. As noted elsewhere, the amount of labeled circulating iodide in each animal is not known; hence, the relative amounts of iodine represented by these two values cannot be determined.

Thyroid fractions (fig. 3 and 4). The principal characteristic of the iodine fraction in the thyroids of the hypophysectomized animals is the low level of radiothyroxine

present at all time-intervals studied. From the first to the 96-hour interval, only 5 to 10 per cent of the labeled iodine taken up in the thyroid gland was in the thyroxine fraction. In the normal rats 15 per cent of the thyroid's I* was thyroxine at 1 hour, and this fraction rose to 20 per cent in 4 hours and 40 per cent in 96 hours. Concurrent with the rise in thyroxine I* in the normal rats was a fall in the diiodotyrosine fraction. At the earliest time-intervals this fraction comprised 75 per cent of the thyroid's I*, but it fell to less than 40 per cent in 96 hours. These are the lowest radiodiiodotyrosine values found in such studies. The inorganic fraction remained for the most part low in both normal and hypophysectomized animals. This is in keeping with previous findings that in the thyroid gland iodide I* is always a minor fraction (1, 2).

Plasma fractions (fig. 5 and 6). At 1 hour, plasma's I* was 95 per cent in the form of iodide in the normal rat, while the thyroxine fraction contained only 2 per cent of plasma's total radioactivity. At 4 hours iodide dropped to 65 per cent while thyroxine rose to 25 per cent. At 96 hours the distribution arrived at was 83 per cent thyroxine and 11 per cent iodide. The diiodotyrosine fraction contained only 5 to 10 per cent of plasma I* at all time-intervals examined.

The striking characteristic of the hypophysectomized rat's plasma was the finding that very little of the initially labeled iodide became converted to thyroxine. The radiothyroxine was always less than 5 per cent of total plasma I*. In contrast with the normal animals, the diiodotyrosine fraction remained higher than thyroxine while by far the greatest part of plasma I* was in the iodide fraction even after 96 hours. At this time 80 per cent of the plasma's initial I* was still present as iodide in the hypophysectomized rats, as contrasted with only 12 per cent in the control animals.

DISCUSSION

The rôle of the thyroid gland in iodine metabolism may be arbitrarily divided into 3 processes: *a*), the gland's removal of iodide from the circulation; *b*), the organic binding of iodide in the gland and perhaps to some extent in other tissues; and, *c*), the release of organic compounds into the bloodstream. These three processes may now be compared in the normal and in the hypophysectomized animals.

At the early hour a much smaller amount of the initially labeled iodide appeared in the thyroid gland of the hypophysectomized than in that of the normal animals. Moreover, the character of the curves depicting the uptake of the initially labeled iodide differed in the two types of animals. In contrast with the normal thyroid gland, which contained over 60 per cent of the injected I* at 4 hours and less than 10 per cent at 96 hours, the thyroid gland of the hypophysectomized rats showed a continuous rise in its content of I* throughout the entire period of observation, but at no time attained a value higher than 25 per cent.

The absolute amounts of total iodine taken up by the glands of these two types of animals cannot be determined from the measurements of radioiodine alone. It would also require measurements of the absolute amounts of iodide initially labeled by the introduction of the 'tracer' dose of iodide. If we assume that the inorganic iodine content of plasma is increased after hypophysectomy, then unit amount of radioactivity will label a larger amount of iodide in the hypophysectomized than in the normal animal. In this case, the difference in the actual or absolute uptake of iodine by the two types of glands is obviously not represented by the two curves depicting the uptake of I*. If, on the other hand, the iodide content of plasma of the hypophysectomized animal is not much different from or even a little lower than that in the normal animal, then the difference shown by the two curves in figure 1 is a reliable

index of the iodine removal in these two conditions. Unfortunately, satisfactory data are not available on the content of inorganic iodine of plasma of hypophysectomized animals, probably because of the lack of suitable analytical procedures for iodine measurements in plasma.

Irrespective of the absolute amounts of iodine taken up by the gland in these two conditions, the results obtained in the present investigation reveal for the first time a qualitative difference in the treatment of iodide by the normal and hypophysectomized rats.[5] Although quantitative comparisons can not be made, the results nevertheless suggest that the conversion of iodide to diiodotyrosine is not interfered with in the hypophysectomized rat, but the conversion of iodide to thyroxine is definitely limited or almost inhibited. Should thyroxine be formed via diiodotyrosine, the results obtained here further indicate that the reaction of diiodotyrosine to thyroxine is almost completely inhibited.

The depressed ability of the thyroid gland of the hypophysectomized rat to make thyroxine is also reflected in the low percentage of plasma's I* found as thyroxine. Almost all of the plasma's I* was present as iodide; that portion present as thyroxine did not exceed 5 per cent at any time during the entire 96 hours of observation.

It is of interest to note that at the 96-hour interval over 80 per cent of the plasma's I* in the normal rat was thyroxine; yet diiodotyrosine was by no means absent from the gland. At this time-interval about 40 per cent of the normal gland's I* was present as diiodotyrosine and only 40 per cent as thyroxine. Moreover, the failure of diiodotyrosine to make its appearance as a large fraction of the plasma's I* at such early intervals as 4, 12 and 36 hours, i.e. at times when the gland's I* is still largely present as diiodotyrosine, suggests that the diiodotyrosine is not normally released into the bloodstream by the thyroid gland.

SUMMARY

The conversion of iodide to diiodotyrosine and thyroxine-like fractions was compared in normal and hypophysectomized rats with radioiodine as indicator.

At the early intervals, the percentage of the administered I* found in the thyroid gland was much less in the hypophysectomized than in the normal rat.

The conversion of iodide to diiodotyrosine is shown to occur readily in the hypophysectomized rat. Approximately 80 per cent of the thyroid's I* was found in the diiodotyrosine-like fraction in the hypophysectomized rat. An appreciable portion of the plasma's I* was also present as diiodotyrosine.

The percentage of the thyroid's I* present as thyroxine was much less in the hypophysectomized than in the normal rat.

---

[5] In a recent report Leblond and Sue (6) conclude, "The extent of decrease in iodine fixation after hypophysectomy . . . indicate that the hypophysis does not influence the specificity of the thyroid activity but regulates the fixation of iodine by controlling to some extent the amount and efficiency of the thyroid tissue."

These workers injected large doses of labeled iodine into their animals. Each rat received 750 micrograms of iodine as iodide. Since the capacity of the gland for converting inorganic iodine to organically bound iodine is limited, it is not surprising that these workers found most of the radioiodine in the thyroid gland in the inorganic form. When 'tracer' doses are used (doses that do not increase the concentration of circulating iodide but yet serve to label the endogenous circulating iodide) it has been shown repeatedly in this laboratory that almost all of the radioiodine deposited in the thyroid gland is organically bound as early as one hour after the introduction of the radioiodine.

As noted in the present investigation, there is a qualitative difference in the treatment of inorganic iodine by glands of the normal and hypophysectomized rats. To reveal such a difference, it is not enough, as Leblond and Sue have done, to divide thyroid I* into organic and inorganic fractions. As shown in the present investigation the difference in the behavior of the glands of these two types of animals lies in the two fractions of organically bound iodine, namely diiodotyrosine and thyroxine. In the glands of both types of animals radioiodide was found at low levels.

In the normal rat the plasma I* is rapidly converted to thyroxine so that at 96 hours about 80 per cent of the plasma's I* is found as thyroxine. In the hypophysectomized rat the percentage of plasma's I* found in the thyroxine-like fraction was negligible throughout the entire 96 hours of observation.

It is suggested that in the hypophysectomized animal the conversion of iodide to diiodotyrosine is not severely interfered with, but that the over-all conversion of iodide to thyroxine is limited.

REFERENCES

1. Morton, M. E., I. Perlman and I. L. Chaikoff: *J. Biol. Chem.* 140: 603. 1941.
2. Perlman, I., M. E. Morton and I. L. Chaikoff: *Endocrinology* 30: 487. 1942.
3. Perlman, I., I. L. Chaikoff and M. E. Morton: *J. Biol. Chem.* 139: 433. 1941.
4. Perlman, I., M. E. Morton and I. L. Chaikoff: *J. Biol. Chem.* 139: 449. 1941.
5. Leblond, C. P., P. Sue and A. Chamorro: *Compt. rend. Soc. de biol.* 133: 540. 1940.
6. Leblond, C. P., and P. Sue: *Am. J. Physiol.* 134: 549. 1941.

PARALLELISM IN THE RESPONSE OF THYROID AND PARATHYROID TO VARIOUS HORMONES AND HORMONE-LIKE SUBSTANCES[1]

HERMAN T. BLUMENTHAL AND LEO LOEB

From the Laboratory of Research Pathology, Oscar Johnson Institute, Washington University School of Medicine

ST. LOUIS, MISSOURI

INVESTIGATIONS concerning the relationship of the parathyroid gland to the other glands of internal secretion have been limited for the most part to certain experiments concerning the effect of hypophysectomy and the effect of the administration of anterior hypophysis on the parathyroid gland. The former procedure was found to diminish or inhibit the proliferative activity of the parathyroid, whereas administration of anterior hypophysis increased it. These earlier experiments were qualitative in nature and the degree of increase or decrease in proliferative activity of the parathyroid under these experimental conditions was not accurately determined; nor was the effect of other endocrine glands than the anterior hypophysis on the activity of the parathyroids studied by more exact means. No definite experiments seem to have been made concerning the possibility that the effect of the administration of anterior pituitary substance upon the parathyroids might be mediated through one or more of the other endocrine glands.

A number of years ago, in some unpublished experiments, it was observed by us that when mitotic activity in the thyroid was increased by the administration of anterior hypophyseal extract there was a corresponding increase in mitotic activity in the parathyroid glands. This suggested the possibility that there might be a more general parallelism in the mode of reaction of the parathyroid and thyroid glands to various conditions. In order to test this possibility it was decided to compare quantitatively, by means of mitotic counts, the effects of certain known stimulating and depressing agents of proliferative activity in the thyroid upon mitotic activity in the parathyroid glands.

MATERIALS AND METHODS

Both male and female guinea pigs, weighing between 150 and 250 gm., were used in these experiments. For the purpose of increasing mitotic activity in the thyroid gland, intraperitoneal injections of acid extract of anterior hypophysis (prepared according to the method of Loeb and Bassett (1), subcutaneous implants of fresh bovine anterior pituitary gland, and feeding of potassium iodide by mouth were used. For the purpose of diminishing mitotic activity in the thyroid gland, the guinea pigs were fed desiccated thyroid substance.

Mitotic counts in the thyroid gland were carried out according to the method used in other investigations from this laboratory; the number of mitoses in every fifth section of the serially sectioned thyroid gland was determined, and this number multiplied by the factor 5 was taken as the total number of mitoses in the gland.

Received for publication December 29, 1941.

[1] These investigations were carried out with the aid of grants from The International Cancer Research Foundation and from the Jane Coffin Childs Memorial Fund for Medical Research.

Mitotic counts in the parathyroids represented the number of mitoses per 10,000 epithelial cells. In order to facilitate the making of these counts there was inserted in the ocular of the microscope an arbitrary rectangular field, which held per field approximately 200 parathyroid cells of a normal 150 to 250-gm. guinea pig. The number of mitoses in 50 fields was counted, as well as the average number of cells per field; the latter figure was found by averaging the cell counts of five fields. From these data the number of mitoses per 10,000 cells was determined and the average number of cells per unit field served also as an index of cell size.

EXPERIMENTS

Mitotic Counts in the Thyroid and Parathyroid Glands of Control Guinea Pigs

(*a*) *Untreated controls.* Twenty-five male and an equal number of female guinea pigs served as normal, untreated controls; the average mitotic count in the thyroid of

TABLE 1. MITOTIC COUNTS IN THYROID AND PARATHYROID GLANDS FOLLOWING ADMINISTRATION OF ANTERIOR HYPOPHYSIS OF CATTLE, POTASSIUM IODIDE AND THYROID SUBSTANCE

Experiment	Thyroid Count			Parathyroid Count			
	No., Sex	Av. No. mitoses/ gland	Av. exper. count / Av. normal count	No., Sex	Av. No. mitoses/10^4 cells	Av. exper. count / Av. normal count	Av. No. cells/unit field
I. Control guinea pigs							
a) Untreated	25♂	146.0 (80–300)		25♂	1.7 (0–5.6)		213.5 (150–285)
	25♀	255.6 (100–560)		25♀	2.2 (0.4–4.9)		205.7 (165–244)
Av.		199.8			2.0		209.6
b) Saline injected	10♀	248.0 (100–410)	0.98	10♀	2.0 (0–3.5)	0.91	186.5 (174–204)
c) Underfed	13♀	41.4 (0–80)	0.16	13♀	0.6 (0–1.4)	0.27	213.9 (150–251)
II. Guinea pigs receiving anterior hypophysis of cattle							
a) Injections of acid extract of bovine anterior hypophysis	10♂	11,955 (1400–32,500)	82.6	16♂	8.0 (1.0–20.0)	4.7	176.0 (120–238)
	20♀	17,822 (1000–58,750)	69.7	34♀	8.3 (1.0–32.3)	3.8	161.2 (140–250)
Av.		15,906	74.0		8.2	4.1	165.9
b) Implants of cattle anterior hypophysis	20♀	11,699 (1250–75,000)	45.8	30♀	6.9 (2.0–16.7)	3.1	187.0 (150–235)
III. Guinea pigs fed potassium iodide	51♂	564 (110–2310)	3.9	51♂	4.4 (0–19.3)	2.6	182.0 (134–226)
IV. Guinea pigs fed desiccated thyroid substance	15♀	46.2 (0–150)	0.2	30♀	1.1 (0–4.6)	0.5	270.1 (175–392)

male guinea pigs was 146.0, while that in females was 255.6; the average for both sexes combined was 199.8. A similar difference between the two sexes was noted also in the parathyroid glands, where the average number of mitoses was 1.7 per 10,000 cells in males and 2.2 per 10,000 cells in females; the average for both sexes was 2.0 (see table 1). The variations in these counts, as indicated by the figures in brackets, could partly be accounted for by the fact that a decrease in mitotic activity occurs in normal guinea pigs with increasing weight (age); thus, some of the males weighing 150 gm. have as high or higher mitotic counts than some females weighing approximately 250 gm. It should be noted, furthermore, that the number of parathyroid cells per unit field was greater in males than in females (213.5 in the former and 205.7 in the latter); this would indicate that, in general, the parathyroid cells are somewhat larger in females than in males of approximately equal weight, although here also there were a few exceptions.

(*b*) *Controls injected with normal saline.* A second group of 10 female guinea pigs received injections of physiological salt solution. The mitotic counts in the thyroid and parathyroid glands did not differ significantly from those in untreated females. However, the number of parathyroid cells per unit field was decreased following the injections of saline, which indicated that there was an increase in the size of the epithelial cells of this gland, perhaps due to the uptake of fluids.

(*c*) *Controls underfed for 6 days.* A third group of 13 female guinea pigs was underfed for a period of 6 days. The purpose of this experiment was to produce a loss in weight comparable to that which frequently follows the injection of acid extract of cattle anterior hypophysis. These guinea pigs lost, on an average, 31.4 gm., or approximately 15 to 20 per cent of their original weight. As can be seen in table 1, underfeeding produced a marked diminution in mitotic activity in both the thyroid and parathyroid glands. The average mitotic count in the thyroid was 41.4, which is 0.16 of that in normal females. The parathyroid count was 0.6 mitoses per 10,000 cells, or 0.27 of that in normal female guinea pigs. In the latter gland there was also an increase in the average number of cells per unit field (213.9 as compared with the normal count of 205.7), indicating a slight decrease in the size of the individual cells.

Effect of Administration of Anterior Hypophysis of Cattle on Mitotic Activity in the Thyroid and Parathyroid Gland

Mitotic counts in the thyroid gland were made in 10 males and 20 females receiv- daily intraperitoneal injections of 1 cc. of acid extract of anterior hypophysis, for periods ranging from 1 to 6 days, and in 20 female guinea pigs receiving subcutaneous implants of one-fourth of a gland of bovine anterior hypophysis daily for 2 days. The guinea pigs treated with acid extract were examined 24 hours after the last injection, while those receiving subcutaneous implants were examined 1, 2, or 3 days following the last implantation. The hypophyseal extract produced a marked increase in mitotic activity in the thyroid gland of both male and female guinea pigs (see table 1). While the number of mitoses in female guinea pigs was on the average higher than in males (17,822 as compared with 11,955), the degree of increase as compared with untreated controls of the corresponding sex was greater in males than in females (82.6 as compared with 69.7). The average mitotic count in both sexes receiving anterior hypophyseal extract was 15,906; this was 74 times greater than that in untreated controls.

Similarly, the implantation of fresh bovine anterior hypophysis in the females produced a marked increase in mitotic proliferation in the thyroid (11,699 mitoses), which was 45.8 times greater than in untreated controls. This form of administration was somewhat less effective than intraperitoneal injections of acid extract of anterior hypophysis.

Parallel results were observed in the parathyroid glands of 16 males and 34 females receiving acid extract and of 30 females receiving subcutaneous implants of cattle anterior hypophysis. The number of injections and implantations, as well as the periods of administration of these substances, was the same as in the guinea pigs in which thyroid counts were made. In a number of instances, mitotic counts were carried out in the thyroid and parathyroid glands of the same guinea pig. In the parathyroid glands, mitotic activity following the injection of acid extract was also slightly higher in females than in males (8.3 per 10,000 cells in females as compared with 8.0 in males), but here, also, the degree of increase as compared with untreated controls of the corresponding sex was greater in males than in females (4.7 as compared with 3.8). The figures showing the number of cells per unit field in the parathyroid gland indicate that, in addition to increased mitotic activity, anterior hypoph-

ysis preparations cause an increase in the size of the individual cells of this gland; this increase in cell size is slightly greater in females than in males.

As in the case of the thyroid, implants of cattle anterior hypophysis increased the mitotic counts in the parathyroid slightly less effectively than injection of acid extract. The average number of mitoses per 10,000 cells in this group was 6.9, an increase of 310 per cent over that in untreated controls. The figures representing the number of cells per unit field indicate an increase in cell size over that of control guinea pigs, but it is less marked than that seen after injections of acid extract. There is, therefore, a definite similarity in the effects of both of these anterior hypophyseal preparations on the thyroids and parathyroids of guinea pigs.

Effect of Feeding Potassium Iodide on Mitotic Activity in the Thyroid and Parathyroid Glands

Fifty-one male guinea pigs were fed either 0.01 gm. or 0.05 gm. of KI daily for periods ranging from 5 to 20 days. There was no constant significant difference in mitotic counts between the two doses. The average count in the thyroid gland for this group was 564 mitoses, which amounts to a 3.9-fold increase of mitoses over that

TABLE 2. THYROID AND PARATHYROID CELL AND MITOSIS COUNTS IN GUINEA PIGS FED KI

No. of Guinea Pigs	No. of Days Fed KI	Aver. No. of Mitoses per Thyroid Gland	Aver. No. of Mitoses per 10^4 Parathyroid Cells	Aver. No. of Cells per Unit Field
15	1–5	336.6	4.7	180
15	7–10	448.6	4.3	190
15	12–15	1012.3	5.4	175
6	20–30	366.6	3.6	185

of male controls. Similarly, the average count in the parathyroid gland under the influence of KI was 4.4 mitoses per 10,000 cells, a number 2.6 times greater than the average mitotic count observed in male controls. The number of parathyroid cells per unit field was 182, indicating an increase in cell size approximately equal to that following the implantation of fresh cattle anterior hypophysis.

In table 2 are shown the mitotic counts arranged according to the length of time during which KI was administered. Gradually the average mitotic count in the thyroid gland increases until a maximum is reached between the 12th and 15th days. This is followed by a regression in mitotic activity when the potassium iodide is fed during a period of 20 to 30 days. In the parathyroid also, a maximum mitotic count is reached between 12 to 15 days, and this maximum also is followed by a decline, but there is here no rise in the mitotic count in the first two periods. The figures representing the average number of parathyroid cells per unit field show the parallelism which exists between the increase in mitotic activity and in the size of the individual cells, the largest cells having been observed after 12 to 15 days of KI feeding and the cells decreasing again in size after feeding of this substance for 20 to 30 days.

The increase in mitotic activity corresponding to the length of time during which administration of KI took place until a maximum of mitotic proliferation occurred after 12 to 15 days is in accord with the experiments reported by Gray and Loeb (2), Rabinovitch (3) and others.

Influence of Thyroid Feeding upon Mitotic Activity in the Thyroid and Parathyroid Glands

Female guinea pigs were fed desiccated thyroid in doses of 0.1, 0.2 and 0.6 grains for periods of 2, 6, 10, 12, 15, 20 and 30 days. We did not arrange the results according to both dosage and length of administration of this substance, because there were only a few experiments in each of these subgroups. Thyroid counts were made in 15 animals and parathyroid counts in 30. There was a marked diminution in mitotic activity in both the thyroid and parathyroid glands (table 1). There was an average mitotic count of 46.2 (0.2 of the normal number in the thyroid) and there was also a decrease in the mitotic count in the parathyroid (1.1 mitoses per 10,000 cells, which is 0.5 of the normal number of mitoses). The average size of the parathyroid cells was very much diminished, as indicated by the average cell count of 270.1 per unit field. If we divide all of these guinea pigs into two groups according to the time of administration of desiccated thyroid substance, the average mitotic count in the thyroid of 7 guinea pigs in the group fed for 2 to 12 days was 53.9, as compared with an average count of 37.4 in the 8 guinea pigs fed for 15 to 30 days. Similarly, the

TABLE 3. COMPARISON OF MITOTIC ACTIVITY IN THE THYROID AND PARATHYROID GLANDS

No. of Guinea Pigs	Thyroid Count		Parathyroid Count	
	Aver. No. of mitoses per gland	Range of mitotic counts	Aver. No. of mitoses per 10^4 cells	Aver. No. of cells per unit field
18	125.5	0–200	0.8(0–4.6)[1]	235.5(185–376)[1]
12	390.4	201–500	1.6(0–5.6)	211.3(150–251)
14	620.3	501–1000	2.4(0.8–3.7)	196.7(140–230)
19	2643.2	1001–5000	7.1(2.0–19.3)	181.4(135–215)
11	13,553.6	over 5000	11.2(2.3–32.3)	168.2(110–235)

[1] Figures in parentheses represent range of variation.

average mitotic count in the parathyroid gland was 1.3 per 10,000 cells in the 19 guinea pigs fed for 2 to 12 days, as compared with 0.7 for the 11 guinea pigs receiving thyroid substance for 15 to 30 days. These results show that thyroid feeding diminishes the mitotic activity in both thyroid and parathyroid glands and that the degree of diminution is greater if feeding is carried out for 15 to 30 days than if it extends over a period of only 2 to 12 days.

From the foregoing experiments it may be concluded that a fargoing parallelism exists between mitotic activity in the thyroid and parathyroid glands; in the latter there appears to be, in addition, an increase in the size of the epithelial cells which accompanies the increase in mitotic proliferation. This parallelism was evident in experiments in which proliferative activity in the thyroid was increased, as well as in others in which it was diminished by various methods; the proliferative activity in the parathyroid gland took a similar course under these different conditions.

The parallelism in the proliferative changes in these glands can be shown in still another way, namely, by grouping the guinea pigs according to the mitotic activity in their thyroid glands, without regard to the treatment which the individual animals had received, and by comparing the mitotic activity in these groups of animals with that in the parathyroid glands of the same animals. The results of this analysis in 74 guinea pigs are shown in table 3.

It may be noted that although in general when the mitotic activity in the thyroid gland is increased, there is an increase also in the proliferative activity and in the size

of the cells in the parathyroid gland; it can be seen from the range of variation indicated by the figures in brackets that there were guinea pigs with relatively low thyroid counts and relatively high parathyroid counts, and others with high thyroid counts and low parathyroid counts. Among the 74 guinea pigs shown in table 3, there were six instances showing variations of this kind (table 4). The reason for these discrepancies is not apparent, but they represent only a very small (8.1 per cent) proportion of the experiments.

TABLE 4. THYROID AND PARATHYROID COUNTS IN INDIVIDUAL GUINEA PIGS IN WHICH THERE WAS A DISCREPANCY BETWEEN THE PROLIFERATIVE ACTIVITY IN THYROIDS AND PARATHYROIDS

No. of Guinea Pig	Average Number of Mitoses in Thyroid Gland	Parathyroid Count	
		Average number of mitoses per 10^4 cells	Average number of cells per unit field
1	190	4.6	185
2	460	5.6	150
3	850	0.8	230
4	1450	2.0	215
5	10,500	3.3	225
6	9650	2.3	335

Experiments were next carried out to determine if the parathyroid glands could be stimulated to increased mitotic activity in the absence of the thyroid glands; the results are shown in *experiments* 1 and 2 of table 5. In thyroidectomized guinea pigs

TABLE 5. EFFECT OF STIMULATING AGENTS ON THE PARATHYROID GLANDS OF COMPLETELY AND PARTIALLY THYROIDECTOMIZED GUINEA PIGS

Experiment	No. of Guinea Pigs	Parathyroid Counts		
			No. mitoses per 10^4 cells	No. cells per unit field
(1) Thyroidectomy 10 mg. KI for 12 days[1]	4		2.9(1.0–4.8)	191.3(165–220)
(2) Thyroidectomy. 1 cc. acid extract of hypophysis for 4 days	2		0.4(0–0.8)	230.0(210–250)
(3) ⅔ of right lobe extirpated; left lobe intact. 1 cc. of acid extract of hypophysis for 4 days	3	Rt. lobe	1.1(1.0–1.3)	178.3(170–190)
		Lt. lobe	4.7(4.3–5.0)	181.0(165–190)
(4) Right lobe completely extirpated; left lobe intact. 1 cc. of acid extract of hypophysis for 4 days	2	Rt. lobe	0.5(0–0.9)	220(210–230)
		Lt. lobe	4.0(3.9–4.2)	197.5(190–205)
(5) ⅔ of each lobe extirpated. 1 cc. of acid extract of hypophysis for 4 days	3	Rt. lobe	2.5	160[2]
		Lt. lobe	4.0(2.6–6.4)	206(203–210)

[1] Remnants of thyroid tissue were found at autopsy.
[2] In only 1 guinea pig was the right parathyroid recovered at autopsy.

fed KI there was evidence of increased mitotic activity in the parathyroid glands, but it was less than in guinea pigs with intact thyroid glands. However, in all four of these guinea pigs some remnants of thyroid tissue were found at the time of examination. In two other thyroidectomized guinea pigs, 1 cc. of acid extract was injected daily for 4 days. In these animals there was no evidence of increased mitotic activity, but on the contrary, the mitotic proliferation was lower than in normal controls. No

remnants of thyroid tissue were found at autopsy in this experiment. In six other experiments of this type we were unable to find the parathyroids at autopsy.

We next attempted to determine whether these results were due to the absence of the thyroid tissue or whether they were due to interference with the blood supply of the parathyroid glands resulting from the operation. In one experiment two-thirds of the right lobe of the thyroid gland was extirpated, leaving a parathyroid gland with the remaining thyroid tissue, while the left lobe was left intact; acid extract was injected subsequently. In the three guinea pigs so treated there was a lower mitotic count in the parathyroid gland on the operated side than on the non-operated side; but even in the latter the count was lower than in guinea pigs with intact thyroids to which acid extract had been administered. In a second experiment the right lobe was completely extirpated, but a parathyroid was replaced approximately in its original location, while the left thyroid remained intact; acid extract of the hypophysis was subsequently injected. Here also the parathyroid of the non-operated side responded to a moderate degree to acid extract, while the gland on the operated side did not. Finally in a last experiment two-thirds of both lobes of the thyroid were removed, leaving a thyroid remnant with an intact parathyroid on each side, and hypophyseal extract was then injected. Three guinea pigs received this treatment, but in only one case were we able to recover the parathyroid on the right side; here it showed a slight proliferative response to the extract, while the opposite side showed a somewhat higher degree of mitotic activity, due principally to one count of 6.4 mitoses per 10,000 cells.

These experiments indicate that the operative procedure incident to thyroidectomy causes a diminution, and, in some cases, actual prevention of the response of the parathyroid glands to stimulating agents. This was further indicated by the fact that in many instances such operative procedures caused visible damage to the parathyroid cells in that they contained pyknotic nuclei and other signs of degenerative change. On the operated side the injury to the parathyroid was more marked, but there was a relative diminution in the proliferative activity even on the non-operated side, which was perhaps due to circulatory changes extending to the side in which the thyroid lobe had been left intact.

DISCUSSION

These experiments demonstrate a remarkable parallelism in mitotic activity of the thyroid and parathyroid glands. Acid extract of anterior hypophysis, which was shown by Loeb and Bassett (1) to produce marked mitotic activity in the thyroid gland, as well as enlargement of the cells of the acinar epithelium, also increases considerably the mitotic activity in the parathyroid gland; implants of fresh cattle anterior pituitary produce similar results but in a somewhat lesser degree. The administration of KI by mouth was found by Gray and Loeb (2), Rabinovitch (3) and others to cause an increase in mitotic activity in the thyroid gland of the guinea pig; in these experiments we have demonstrated that it acts similarly on the parathyroid gland. We have shown, furthermore, that associated with the increase in mitotic activity in the parathyroid gland there is usually an increase in the size of the parathyroid cells, and thus both hyperplasia and hypertrophy result. Rabinovitch (4) observed that underfeeding produces a diminution in mitotic activity in the thyroid gland of the guinea pig; we have been able to verify this observation and to note that a similar effect is exerted on the parathyroid gland. Similarly, Loeb (5) has reported that thyroid feeding produces a diminution in proliferative activity in the thyroid gland and a flattening of the epithelial cells; this treatment correspondingly produces a diminution in mitotic activity as well as in cell size in the parathyroid gland.

According to Collip (6) the existence of a parathyrotropic hormone of the anterior hypophysis is doubtful. However, the evidence in favor of the occurrence of such a hormone is based upon certain clinical and experimental observations. The clinical evidence rests on reports by Hadfield and Rogers (7), Gerstel (8), Husslein (9) and others, who have noted cases in which parathyroid adenomas occurred in association with hypophyseal tumors. Experimental investigations were of two kinds: *a*), on the effects of hypophysectomy on the parathyroid glands; and *b*), on the stimulation of the parathyroids by the administration of anterior hypophyseal extracts.

As to the changes after hypophysectomy, Koster (10), Houssay and Sammartino (11), and Smith (12) have reported that this procedure results in an atrophy of the parathyroid glands. On the other hand, Aschner (13) could find no significant change in the parathyroids following hypophysectomy. Increased proliferative activity resulting from the injection of extracts of anterior hypophysis has been reported also by Anselmino, Herold and Hoffman (14), who found enlargement of the epithelial cells, follicle formation and hyperemia, without enlargement of the total volume of the gland, and by Hertz and Kranes (15), who observed an increase both in size and in the number of mitoses in the epithelium of the parathyroid.

Zondek (16) has shown that the thyrotropic and parathyrotropic function of the pituitary in rats and chickens is stimulated by long-term estrin injections, and more recently, Nathanson, Brues and Rawson (17) have observed that injections of testosterone propionate in rats produces increased proliferative activity in the thyroid and parathyroid glands of these animals. The latter investigators note that there are two possible mechanisms underlying this reaction, namely, *a*, the direct effect of the hormone, and, *b*, stimulation of these two glands through the anterior pituitary or some other gland of internal secretion. It is their opinion that it is probably the anterior hypophysis which is stimulated by the injections of testosterone, since they were previously able to show that testosterone stimulates the ovary by way of the anterior pituitary even in the absence of the adrenals.

As to the remarkable parallelism in the normal proliferative activities in the thyroid and parathyroid glands and in the effects of experimentally administered hormones and of potassium iodide on these two glands, two possibilities exist. *a*) The thyroid and parathyroid glands both may be affected by these substances either directly or by way of the anterior pituitary. *b*) Only the thyroid may be directly affected and the increased activity of the thyroid may secondarily stimulate the parathyroid to a similar degree of proliferation. This latter possibility would imply that a decrease in normal thyroid activity, caused by feeding of thyroid substance, would affect in a corresponding manner the parathyroid. We attempted to determine which of these two possibilities applies by administering thyroid-stimulating hypophyseal extract to thyroidectomized animals. After complete thyroid extirpation, the parathyroid mitotic count which usually is markedly increased following injection of hypophyseal extract, was reduced. But it is possible that this was a secondary effect, probably caused by interference with the circulation, for in partially thyroidectomized animals the parathyroid mitotic count was diminished to a greater extent on the side where the gland had been extirpated. Under these conditions it has been impossible so far to decide definitely the question as to the mechanism underlying the remarkable parallelism between these reactions of thyroid and parathyroid. However, we believe it is more probable that the response of the thyroid and that of the parathyroid to various hormones and hormone-like substances are similar, than that the parathyroid proliferation is determined by a substance transmitted by the thyroid to the parathyroid.

SUMMARY

1. Mitotic counts in the thyroid and parathyroid glands show that in guinea pigs weighing between 150 and 250 grams, the proliferative activity in both glands is greater in females than in males.

2. The administration of acid extract of anterior hypophysis, hypophyseal implants, and feeding of KI results in increased mitotic activity in both the thyroid and parathyroid glands. Hypophyseal extract produces the greatest degree of proliferation and is followed by implants of anterior hypophysis and KI in order of diminishing effect, and this order applies equally to thyroid and parathyroid.

3. Underfeeding as well as the administration of thyroid substance results in a marked diminution of mitotic activity in both the thyroid and parathyroid glands.

4. In both glands, increased growth under the influence of stimulating substances consists in the processes of hyperplasia as well as hypertrophy.

5. It is uncertain how far this parallelism in the action of various hormones and of iodine acting on the thyroid and parathyroid is a direct one or an indirect one mediated by the anterior hypophysis.

REFERENCES

1. Loeb, L., and R. B. Bassett: *Proc. Exper. Biol. & Med.* 27: 490. 1930.
2. Gray, S. H., and L. Loeb: *Am. J. Path.* 4: 257. 1928.
3. Rabinovitch, J.: *Am. J. Path.* 4: 601. 1928.
4. Rabinovitch, J.: *Am. J. Path.* 5: 87. 1929.
5. Loeb, L.: *Am. J. Surg.* 7: 12. 1929.
6. Collip, J. B.: *J. Am. Med. Assoc.* 115: 2073. 1940.
7. Hadfield, G., and H. Rogers: *J. Path. & Bact.* 35: 259. 1932.
8. Gerstel, G.: *Ztschr. f. Path.* 52: 485. 1938.
9. Husslein, J.: *Beitr. z. klin. Chir.* 169: 276. 1939.
10. Koster, S.: *Arch. f.d. ges. Physiol.* 224: 212. 1930.
11. Houssay, B. A., and R. Sammartino: *Compt. rend. Soc. de biol.* 114: 729. 1932.
12. Smith, P. E.: *J. Am. Med. Assoc.* 88: 158. 1927.
13. Aschner, B.: *Arch. f.d. ges. Physiol.* 146: 1. 1912.
14. Anselmino, K. J., L. Herold and F. Hoffman: *Ztschr. f.d. ges. exper. Med.* 97: 51. 1935.
15. Hertz, S., and A. Kranes: *Endocrinology* 18: 350. 1934.
16. Zondek, B.: *Lancet* 2: 842. 1936.
17. Nathanson, I. T., A. M. Brues and R. E. Rawson: *Proc. Soc. Exper. Biol. & Med.* 43: 737. 1940

FURTHER INVESTIGATIONS ON THE PROLIFERATIVE ACTIVITY OF THE THYROID GLAND OF THE FEMALE GUINEA PIG DURING THE SEXUAL CYCLE[1]

K. S. CHOUKE AND HERMAN T. BLUMENTHAL

From the Laboratory of Research Pathology, Oscar Johnson Institute, Washington University School of Medicine

ST. LOUIS, MISSOURI

and the Department of Anatomy, The Graduate School of Medicine, University of Pennsylvania

PHILADELPHIA, PENNSYLVANIA

IN A PREVIOUS PAPER it was shown by Chouke, Friedman and Loeb (1) that in the female guinea pig, during the sexual cycle, the proliferative activity of the thyroid gland, as determined by mitotic counts, is greater than in males of a corresponding age. It was also shown that the mitotic proliferation of the thyroid gland undergoes cycles which are correlated with the sexual cycle, since mitotic activity was lower during the second part of the cycle and during the follicular phase than during the luteal phase. Furthermore, the results seemed to indicate that the highest degree of mitotic activity was reached on approximately the fifth day following estrus, although this was not definite, since the total number of female guinea pigs examined was only 57 and some of the mitotic counts made on the fifth and sixth days after estrus were as low or lower than counts carried out at other periods in the sexual cycle.

It was therefore thought advisable to make additional counts in female guinea pigs at various stages in the estrous cycle in order to determine more definitely whether cyclic changes take place in the proliferative activity of the thyroid gland during the sexual cycle and at which period the maximum occurs. To the 57 guinea pigs studied previously we now add the mitotic counts of 112 examined at various stages in the sexual cycle, as well as those of 25 males serving as controls. The male guinea pigs ranged in weight from 305 to 690 gm., with an average weight of 487 gm., while the range in weight of the females was between 305 and 665 gm., with an average of 441 gm. Fifteen of the 25 males (60%) and 86 of the 112 females (76.8%) weighed between 300 and 500 gm., while only 3 males and 8 females weighed more than 600 gm. Diet, room temperature and time of autopsy were the same in all animals. The time of estrus was determined by means of vaginal smears in some cases and in others by the sexual response toward males in which the ductus deferens had been ligated. In the early part of these investigations mitoses were counted in every tenth section; later, every fifth section was counted because we found that this procedure increased, to a certain degree, the accuracy of the counts. This latter method was used also in determining differences which existed in the right and left lobes of the thyroid gland in the same animal.

Received for publication December 29, 1941.

[1] These investigations were carried out with the aid of grants from The International Cancer Research Foundation and from the Jane Coffin Childs Memorial Fund for Medical Research.

RESULTS

In table 1, all of the data of the present experiments, as well as those of Chouke, Friedman and Loeb are combined. It can be seen that the average mitotic counts in guinea pigs examined during the first six days of the sexual cycle were higher than those taken during the latter part of the cycle; the averages of these two groups were 412.0 for the first six days and 170.1 for the second part of the cycle. While the minima in the counts were about the same in both periods, the maxima reached were much higher during the first part of the cycle and especially during the fourth to sixth days, when they reached their peak. The average counts in both these groups were higher than in the control males.

TABLE 1. MITOTIC COUNTS IN THYROID GLAND OF THE FEMALE GUINEA PIG DURING THE ESTROUS CYCLE

Days after estrus	Number of guinea pigs	Average number of mitoses per thyroid gland		Range of variation
0	30	134.7		0– 363
1– 3	24	397.9		20–1874
1– 4	35	495.2		20–2415
5	31	470.1	Av. 412.0	0–5784
6	35	514.9		0–3452
4– 6	77	524.0		0–5784
7–10	27	93.6		0– 434
11–14	11	203.2	Av. 170.1	40– 515
Male controls	25	62.0		0– 220

However, the figures in the last column show that the individual variations in the counts were very great, and for this reason it was decided to subject these figures to statistical analysis; the results of this are shown in table 2. It may be noted that there is a very marked significant difference between counts made on males and those in females examined at estrus, while the difference between the counts in males and those in females examined at the time of lowest mitotic activity, namely at 7 to 10 days after estrus, does show a slight but still significant difference. Further, the increase in mitotic activity observed during the first six days after estrus is significant. There is also statistical evidence that there is a gradual increase in mitotic activity

TABLE 2. A COMPARISON OF THE STATISTICAL SIGNIFICANCE BETWEEN THE MITOTIC COUNTS IN VARIOUS GROUPS OF GUINEA PIGS (TABLE 1) AT DIFFERENT PERIODS IN THE ESTROUS CYCLE

Groups compared	Factor of statistical significance[1]
Male controls and 0 days after estrous	10.4
Male controls and 7–10 days after estrous	2.8
0 and 1–3 days after estrous	6.3
0 and 1–4 days after estrous	5.8
0 and 5 days after estrous	3.2
0 and 6 days after estrous	4.7
1–3 and 4–6 days after estrous	8.5
5 and 6 days after estrous	1.9
0 and 7–10 days after estrous	9.1
0 and 11–14 days after estrous	2.9

[1] The factor of statistical significance was calculated according to the relationship, $A_1 - A_2/SE_{A1} - SE_{A2}$, in which A represents the average for a given group and SE the standard error for the corresponding group. If this factor is less than 2 there is no significant difference between the two groups compared, while if it is greater than 3 it is "almost certainly" significant. A factor between 2 and 3 indicates a probability greater than merely a chance phenomenon.

during the first six days of the estrous cycle, with a maximum being reached between the fourth and sixth days, since the increase in the number of mitoses observed at 1 to 3 days after estrus over those observed at the time of heat is significant, as is also the increase in the number of mitoses observed during the fourth to the sixth day of the cycle over that seen during the first three days after estrus. Following this maximum in mitotic activity there is reached a low point at 7 to 10 days after estrus; a comparison of the counts at this time with those observed at the time of estrus shows that this diminution is a markedly significant one. On the other hand, there is only a slight difference between the finding at 11 to 14 days after estrus and those at the time of heat.

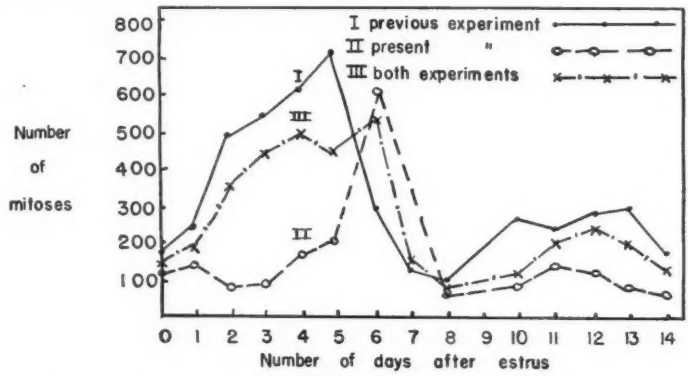


Fig. 1. Curves representing the average mitotic activity of the thyroid gland during the sexual cycle in two series of experiments

The variations in proliferative activity of the thyroid gland during the sexual cycle in the guinea pig are given in figure 1, in which there are three curves representing mitotic counts at the various stages following estrus: *a*), in the experiments of Chouke, Friedman and Loeb; *b*), in the present additional experiments; and *c*), in the combined experiments. In all three curves there is a higher level reached during the first 6 days than during the second part of the estrous cycle, extending from the seventh to the fourteenth day. The peak in the curve is attained between the fourth and sixth days, being reached on the fifth day in the earlier experiments and on the sixth day in the second series.

During the course of these experiments it was decided to compare the mitotic counts in the right and left lobes of the thyroid gland in a number of guinea pigs, in order to determine whether the accuracy of the counts would be increased by counting mitoses in both lobes and whether a serious error might be introduced if the counts of only the right or left lobe were considered. Such a comparison was also indicated because it can be seen with the naked eye that, as a rule, in the guinea pig the left lobe of the thyroid gland is larger than the right lobe. The counts in both lobes are compared in table 3; in 13 of the 16 female guinea pigs studied the left lobe contained a greater number of mitoses than the right lobe. The average count in the left lobe was 87.5 mitoses, while that in the right lobe was 61.3; the average difference between the counts in the two lobes was 26.2, and the difference ranged from −20 to 90 mitoses; there were three cases in which the right lobe contained more mitoses than the left one. While there is thus a definite difference between the counts in

right and left lobes, this difference is relatively small and it does not invalidate the counts in a single lobe.

DISCUSSION

In these investigations we have been able, on the basis of a large number of additional experiments, to substantiate the observation of Chouke, Friedman and Loeb that the mitotic count in the thyroid gland is about three times higher in females than in males; in the present experiments it was found to be approximately four times greater. The possible relationship of the increased thyroid proliferative activity in female guinea pigs during the sexual cycle to the greater readiness with which hyper-

TABLE 3. COMPARISON BETWEEN MITOTIC COUNTS IN RIGHT AND LEFT THYROID LOBES IN THE SAME GUINEA PIG[1]

Guinea pig	Mitotic count in left lobe	Mitotic count in right lobe	Difference in count between coth lobes (L−R)
1	20	20	0
2	40	60	−20
3	50	20	30
4	50	70	−20
5	60	10	50
6	70	20	50
7	70	60	10
8	90	40	50
9	90	90	0
10	100	50	50
11	100	70	30
12	110	60	50
13	110	100	10
14	120	140	−20
15	130	40	90
16	190	130	60
Average Counts	87.5	61.3	26.2

[1] These counts were all in female guinea pigs.

thyroidism occurs in this sex in humans has been mentioned previously (1). We also found that there are cyclic changes in the degree of mitotic activity during the estrous cycle, which correspond in point of time to changes which occur in the ovaries. Mitotic activity appears to be greatest during the luteal phase of the cycle; it then diminishes during the period when the corpus luteum undergoes retrogression, and rises somewhat as the follicles again approach maturation, although it is still considerably lower at this time than during the period when the corpus luteum is fully active.

While in the former series of experiments the maximum in the thyroid counts was found on the fifth day of the cycle, in this series it was the sixth day which showed the peak; but if we compare the averages of all of the counts, the differences between those on the third, fourth, as well as on the fifth and sixth days are very slight, although a slightly greater elevation in the curve between the fifth and sixth days still remains.

However, while there is thus a definite characteristic representing the averages in the counts on the various days of the sexual cycle, nevertheless there are very great differences in the individual counts at all periods of the cycle. These variations are superimposed upon the regularities in the averages of the counts. They could be explained if we assume that in addition to the cyclic function of the ovary, some other

factors influence to a certain degree the mitotic count in the thyroid gland, and that these two sets of factors may interfere with each other; or if we assume that in order to make the action of the sex hormones effective another factor has to cooperate with the former and that the action of the second factor shows a certain variability. In either of these two ways the irregularities in the counts which exist at all periods of the cycle might perhaps be explained.

While, as stated, the highest mitotic counts were obtained during the luteal phase of the sexual cycle, there was an elevation in the number of mitoses also in the second phase and, therefore, throughout the whole period of the cycle and even between the seventh and tenth days, when the average counts were lowest, as compared with the average mitotic counts in the thyroid gland of adult males. If we tentatively attribute the rise in proliferative activity of the thyroid gland in female guinea pigs during the sexual cycle to the action of hormones, we would have to assume that besides luteal hormone, which would then possess the most marked action, estrogen also would increase the mitotic count; however, it will be necessary to test this hypothesis in further investigations. Such a conclusion would agree with the finding of Zondek (2) that the injection of estrin in rats and in chickens stimulated the thyroid gland to increased function.

We have been able to show that in the left lobe of the thyroid gland, as a rule, the number of mitoses is greater than in the right lobe. We were led to investigate the possibility of such a relationship by the observation of Loeb (3) that in the guinea pig, usually the left lobe appears grossly larger than the right. The larger mitotic count in the left lobe may be an indication either of a more abundant blood supply resulting in a better general nourishment or in a richer supply of thyroid stimulating substances to the left lobe, or it may be correlated with the greater number of cells in this lobe, and the occurrence of mitoses in a given number of cells might then be the same in both lobes. This point needs further investigation. It would also be interesting to determine whether or not the difference between the two lobes becomes more marked when the thyroid activity is greatly increased.

SUMMARY

In female guinea pigs during the sexual cycle the proliferative activity of the thyroid gland is greater than in male animals of corresponding weight and presumably also of corresponding age.

Mitotic activity in the thyroid gland of the female guinea pig is greater during the first six days of the estrous cycle than during succeeding periods. Following the peak in cell proliferation during the first six days, and especially during the fourth, fifth and sixth days of the cycle, a decrease to a minimum occurs by about the tenth day, after which period the number of mitoses again shows a gradual rise.

There is some indication that mitotic activity reaches its peak on the fourth, fifth and sixth days of the estrous cycle; however, there occur considerable individual variations at all periods of the cycle which suggest that factors which are mutually antagonistic may perhaps influence the mitotic count.

The total number of mitoses in the left lobe of the thyroid gland in individual guinea pigs is generally higher than that in the right lobe.

REFERENCES

1. Chouke, K. S., Hilda Friedman and L. Loeb: *Anat. Rec.* 63: 131. 1935.
2. Zondek, B.: *Lancet* 2: 842. 1936.
3. Loeb, L.: Personal Communication.

NOTES AND COMMENTS

MITOSIS IN THE ANTERIOR PITUITARY OF THE MONKEY

MOST GENERAL DESCRIPTIONS of the histology of the anterior pituitary gland of mammals still convey the impression that mitoses are exceedingly rare in the differentiated gland. The natural inference is that any shift in the relative proportions of cells present must be due to transitions from one cell type to another. This concept is reinforced by the more extensive generalization that highly specialized cells do not divide mitotically. This latter generalization stems from the fact that usually an environmental situation which favors cytological differentiation is unfavorable for cellular proliferation. However, there is an increasing body of evidence which clearly indicates that under a changed environment or upon appropriate stimulation many highly specialized cells will proliferate mitotically, especially if the type of differentiation does not result in the production of natural barriers to such activity.[1]

It is true that in the recent literature on the pituitary gland reference is most frequently made to the occurrence of mitosis in the chromophobic cells but there are also many descriptions of dividing acidophiles under normal and experimental conditions in both growing and adult mammals. Observations on dividing basophiles are more rare. However, Pomerat[2] has recently described a great increase in the number of dividing basophiles in the male rat following castration. The mitotic picture in this instance was exaggerated by previous treatment of the animals with colchicine.

The present report is based on a single pituitary from a female monkey obtained through the kindness of Dr. Carl G. Hartman who has reported the pertinent data on this animal in a recent publication,[3] (pp. 464–5). "Monkey 602, a 4040 gm. female, was received on March 3, 1938. Cycles after September 15 were 25, 30, 27, 21 days in length. She was castrated on March 2, 1939 and the ovaries were found to be 'empty.' After priming with 100 R.U. of Amniotin daily, from March 17 to 29, 25 R.U. Amniotin plus 50 mg. testosterone was injected daily (except on 2 Sundays) for 17 days (total, .0750 gm. testosterone)! Colchicine was injected at 10:00 A.M. April 20th, 5 days after the last injection, and the animal was killed at 4:30 P.M. The (uterine) mucosa was of medium thickness, the surface epithelium low, with roundish single-ranked nuclei, still, however, showing numerous mitoses; the basal glands show more marked estrogenic effects than those of the functionalis, whose nuclei were in single rows with basal clear zone (glycogen?) below them in some cases."

The pituitary gland was fixed in formalin-corrosive sublimate and stained with the Heindenhain's azan method.[4] This is not an ideal technique for the demonstration of mitoses, especially in acidophiles, since the granules as well as the chromosomes stain intensely red with azocarmine. Mitoses may be more readily overlooked than in preparations in which the tissue has been pre-stained with Ehrlich's or Delafield's hematoxylin before using the trichrome method.

A total of 513 mitoses were recorded in 9 sections uniformly spaced throughout a series of 691 sections cut 4 micra thick. Besides these counts used in the calculations, random counts were made from intervening sections to determine if there was any extensive variation in the distribution of mitoses. There proved to be little tendency for localization of mitotic activity. On the basis of the data a rough calculation indicates that there are about 39,000 mitoses in this anterior pituitary gland. However, it must be kept in mind that this is a

Received for publication January 7, 1942.

[1] DAWSON, A. B.: *Growth suppl.* 4: 91. 1940.

[2] POMERAT, G. R.: *Am. J. Anat.* 69: 89. 1941.

[3] HARTMAN, C. G.: *Endocrinology* 26: 449. 1940.

[4] DAWSON, A. B., AND H. B. FRIEDGOOD: *Stain Technol.* 13: 17. 1938.

cumulative effect due to the colchicine treatment which usually arrests mitosis in early metaphase, or may even possibly stimulate certain prepared cells to embark upon mitosis.

Mitoses were almost entirely restricted to acidophiles and chromophobes. Only two basophiles were seen undergoing division. All of the dividing acidophiles were of the coarsely granulated type reacting specifically with azocarmine.[5] The ordinary acidophiles staining with orange G were less numerous and compressed, and possessed pyknotic nuclei.

The percentages of all cells in the different phases of mitosis are as follows: early prophase, 3.5; late prophase, 1.9; metaphase (normal and modified) 91.2; anaphase, 2.5; telophase, 1.1. Following treatment with colchicine the metaphase figures are usually abnormal with spindle suppression and atypically grouped chromosomes. In the present instance typical metaphases were rare, constituting only 0.7 per cent of all mitoses observed. In all other cells, including both acidophiles and chromophobes, the chromosomes of metaphase were either clumped into a compact mass (57.0% of all mitoses) or widely scattered (35.5% of all mitoses). In the chromophobes both the clumped and scattered chromosomes tended to occupy the center of the cell while in the acidophiles the clumped chromosomes frequently occupied an extreme eccentric position and the scattered chromosomes were often distributed about the extreme periphery of the cell.

The varying position of the chromosomes in the acidophiles may be correlated with the duration of the period of arrest induced by the colchicine since in the more atypical cell patterns the acidophilic granules were no longer uniformly distributed throughout the cytoplasm but were either grouped into irregular clusters or massed toward the center of the cell.

The capacity of acidophiles to divide by mitoses did not appear to be influenced by the density of the granulation, since mitoses appeared to be distributed more or less at random among densely, medium, and lightly granulated cells as well as chromophobes (25.5% in densely, 24.9% in medium, 30.3% in lightly granulated and 19.3% in chromophobes). The proportion of dividing chromophobes is surprisingly small and appears to be correlated with the reduced number of chromophobic cells in this pituitary following the treatment with estrogen and testosterone. The induced increase in the number of carminophile-acidophile cells is associated with a decrease in number of both ordinary acidophiles and chromophobes.

It also seems probable that the chromophobes observed in mitosis are precursors of acidophiles since basophiles are seldom intermingled with acidophiles but are restricted to definite antero-ventral areas of the pars distalis.[5] In these restricted areas, in which basophiles predominate, chromophobes and basophiles rarely exhibited mitosis.

Discussion. The literature on the occurrence of mitosis in the anterior pituitary has been reviewed recently by Pomerat.[2] The specific influences of estrogen and testosterone on mitosis have not been extensively studied. Wolfe and Wright[6] have found that in female rats a marked increase in mitosis was obtained following injection with estrin. A great majority of these were found in chromophobes although a few were found in eosinophiles. Mitosis was highest after 10 days of treatment (normal 1.2 mitoses per section; treated, 36.6 mitoses per section), somewhat reduced after 30 days (26.1 mitoses per section) and greatly reduced after 80 days (10.1 mitoses per section). These authors also record a complete degranulation of basophiles and a great reduction in the number of acidophiles. This was not true of the monkey pituitary.

Injections of testosterone propionate apparently do not exert a comparable influence on mitosis in the pituitary of the female rat. Wolfe and Hamilton[7] treated 25-day old animals for periods of 10 and 20 days. There was no hypertrophy of the pituitary, such as occurs in animals receiving estrin. The pituitary weights of the experimental rats were either slightly less or the same as those of the controls, never greater. Mitotic figures were considerably more abundant in the control rats, both in acidophiles and chromophobes. However, the difference was not great enough to conclude that injection of the male hormone inhibited mitotic division.

There are no records on the amount of mitosis normally present in the monkey pituitary.

---

[5] Dawson, A. B.: *Anat. Rec. suppl.* 2, 73: 63. 1939.
[6] Wolfe, J. M., and A. W. Wright: *Endocrinology* 23: 200. 1938.
[7] Wolfe, J. M., and J. B. Hamilton: *Endocrinology* 25: 572. 1939.

However, in a study of many pituitaries obtained through courtesy of Dr. F. L. Hisaw, I have found mitoses to be exceedingly rare. Accordingly, it seems that the present case represents an instance of marked stimulation of mitosis and that by some happy circumstance the dosage and length of treatment apparently approached optimum. Previous work on the female rat suggests that estrogen rather than testosterone is more effective in producing changes in the pituitary that are accompanied by increased mitotic activity.

Summary. A remarkable number of mitoses were observed in the anterior pituitary of a female monkey, obtained through the kindness of Dr. Carl G. Hartman. This monkey received 100 R.U. of an estrogen* daily for 13 days; 25 R.U. of the same estrogen plus 50 mg. of testosterone propionate daily for 16 days, and colchicine 6½ hours before autopsy. A total of 513 mitoses were counted in 9 sections uniformly spaced through a series of 691 sections. It is calculated that there were about 39,000 mitoses in this anterior pituitary. The mitoses were almost entirely restricted to acidophiles and chromophobes. Two basophiles were seen in mitosis. Over 80 per cent of all dividing cells were acidophiles with the mitoses distributed among densely, medium, and lightly granulated cells. All dividing acidophiles were of the coarsely granulated type, reacting specifically with azocarmine. This example furnishes additional evidence that the cellular population of the anterior pituitary in a mature animal is not static and that the cells will proliferate by indirect division following appropriate stimulation.

ALDEN B. DAWSON

Biological Laboratories
Harvard University
Cambridge, Massachusetts

* Estrogen in the form of Amniotin, E. R. Squibb & Son, New Brunswick, New Jersey.

NOTES ON THE COLORIMETRIC ESTIMATION OF 17-KETOSTEROIDS IN URINE

Comparison of MgO and Girard's reagent for purifying urinary androgen extracts. In order to measure colorimetrically the quantity of 17-ketosteroids in urine by the Zimmerman m-dinitrobenzene reaction with accuracy it is necessary to have a highly purified androgen fraction. The best purifications have been accomplished by means of the Girard reagents as in the method of Talbot, Butler and MacLachlan[1] and the MgO adsorption technique of Baumann and Metzger.[2] Even though the urinary androgens are separated with a Girard reagent, some impurities remain which react with m-dinitrobenzene to give results that are rather high. Color filters correct for only part of the interfering chromogens. A comparison of the degree of purification by MgO and Girard's reagent in six urine extracts has been made using a Klett colorimeter and a 55 Wratten color filter.

TABLE 1. PURIFICATION OF URINARY ANDROGEN EXTRACTS

Urine Extract	MgO	Girard's Reagent T	Capon Assay
	mg./day	*mg./day*	*mg./day*
A	2.2	4.1	
Mi	2.1	3.3	
R	4.2	8.6	
E	7.3	12.1	2.3
J	9.6	14.3	3.6
Mu	10.2	16.1	7.9

Received for publication January 20, 1942.

[1] TALBOT, N. B., A. M. BUTLER AND E. MACLACHLAN: *J. Biol. Chem.* 132: 595. 1940.

[2] BAUMANN, E. J., AND N. METZGER: *Endocrinology* 27: 664. 1940.

In most instances purification with the Girard reagent yielded values 50 to 100 per cent above those given by MgO.

Comparative chromogenic values of androsterone, 3-α-hydroxyetiocholanone-17 and dehydroisoandrosterone with m-dinitrobenzene. The principal steroids in the androgen fraction of normal and most specimens of the pathological human urines are the α-ketosteroids, androsterone and 3-α-hydroxyetiocholanone-17, each of which comprises 40 to 45 per cent of the total[3] and the β-ketosteroids, dehydroisoandrosterone, and isoandrosterone[4,5] which make up 2 to 15 per cent of the total. The color produced with m-dinitrobenzene and alkali by

TABLE 2. PHYSICAL CONSTANTS OF PREPARATIONS OF ETIOCHOLANONE[1]

Preparation	Transition pt. °C.	Melting Point, °C.		$(\alpha)_D$	Color equivalent Androsterone = 100%
		Block	Capillary tube		
Urine etiocholanone, 1	140	146–147			98.6
Urine etiocholanone, 2	142–143	145–150		+104.5	101.5
Synthetic etiocholanone	136–140	149–151	151–153 corr.	+106	99.9
Synthetic etiocholanone given by Ruzicka			150–151 corr.	+104.7	

[1] The micro melting points were made on a Johns block. The color was developed in 100 per cent ethanol.

androsterone and dehydroisoandrosterone has been found to be identical in quality and intensity by Langstroth and Talbot,[6] a fact that we can confirm. Since 3-α-hydroxyetiocholanone-17 comprises so large a part of the total urinary androgens, we wished to know if it also would yield an equal amount of colored substance; as might be expected from its structure, we found that it does. Two specimens were separated from mixed human urines by chromatography but the melting points were low compared to that given by Ruzicka et al.[7] for material prepared from epicoprosterol. Therefore, some etiocholanone was prepared by Ruzicka's method for comparison. Mixed melting points of the natural with the synthetic substance showed them to be identical. With m-dinitrobenzene and KOH, all specimens produced an equal amount of colored compound which was the same as that given by equal weights of androsterone, both in quality and intensity, when compared in a colorimeter without the use of color filters. Physical constants of the various preparations are given in table 2.

EMIL J. BAUMANN
NANNETTE METZGER
DAVID B. SPRINSON

From the Laboratory
Division of Montefiore
Hospital, New York City

[3] CALLOW, N. H., AND R. K. CALLOW: *Biochem. J.* 34: 276. 1940.
[4] BUTLER, G. C., AND G. F. MARRIAN: *J. Biol. Chem.* 124: 237. 1938.
[5] PEARLMAN, W. H.: *J. Biol. Chem.* 136: 807. 1940.
[6] LANGSTROTH, G. O., AND N. B. TALBOT: *J. Biol. Chem.* 129: 759. 1939.
[7] RUZICKA, L., M. W. GOLDBERG, J. MEYER, H. BRÜNGGER AND E. EICHENBERGER: Helv. chim. acta 17: 1405. 1934.

ADRENALECTOMY AND RESPONSES OF THE NICTITATING MEMBRANE TO CERVICAL SYMPATHETIC STIMULATION

A CRITICAL RELATION between adreno-cortical function and the integrity of certain neuromuscular mechanisms has been suggested by Secker,[1] based upon the observation that adrenalectomy in the male cat resulted in rapid loss by the superior cervical sympathetic and the mesenteric nerves of functional control over their respective effector structures. The loss presumably occurred through dissipation of a neuro-myal transmitting substance. As a corollary to the failure in transmission, it was found that judicious administration of an adrenal cortical hormone preparation promptly effected a restoration. Hence a cortical principle appeared to be concerned in formation of the transmitting agent.

This finding appeared especially striking in view of some current concepts concerning the cause of collapse in adreno-cortical insufficiency (e.g., Swingle et al).[2] It is noteworthy, therefore, that Secker's observations failed of substantiation at the hands of Cleghorn and his associates.[3,4] Their experiments differed, however, in that chronic saltor hormone-maintained preparations were employed, whereas the original report was based upon acute experiments. In view of the disparity in results, and of the possibility that the two types of experimental material may actually differ in certain respects, additional observations on the acute preparation are presented.

Experimental procedure. Cats were anesthetized with urethane, 1 gm. per kg. by tube, or sodium pentobarbital, 30 mg. per kg. intraperitoneally. This was followed by tracheotomy and extensive exposure of one vago-sympathetic trunk in the neck. The trunk was cut just above the thorax, the vagal component sectioned again at its ganglion, and the nerve drawn into contact with electrodes in a closed tube so devised as to maintain moisture and viability.[5] The nerve so handled, indeed, proved capable of surviving several hours with no further attention. Blood pressures, when determined, were recorded from a femoral artery in the usual manner. The nictitating membrane was attached by silk thread and pulley wheels to a weighted (5 gm.) heart lever for kymographic recording.

For stimulation a Harvard inductorium driven by a 6-volt storage battery provided the current, adjusted to give at the outset just supramaximal faradic stimulation. In a few experiments a series of normal nictitating-membrane responses were obtained prior to adrenalectomy. Since the operation produced no appreciable change, however, it was performed in the majority of instances 30 to 360 minutes before stimulation was begun. The cervical sympathetic usually was stimulated 30 seconds, at 60-second intervals, for periods up to several hours; occasionally the routine was interrupted by 6-minute periods of continuous stimulation.

Results. Data were obtained from 14 cat preparations, 10 males and 4 females, and are summarized in table 1. With two exceptions, the membrane showed no significant failure in response. Total stimulation periods following adrenalectomy ranged from 12 to 95 minutes, and several animals were practically moribund, usually by intentional insult, at termination of experiment.

Because of the sex differences reported,[1] two females, *cat 9* and *cat 15*, were oöphorectomized at the time of adrenal removal. *Cat 16*, male, was obviously a long-time castrate, as judged at autopsy. Two of these three animals failed to show a failure of response of the nictitating membrane, in the face of stimulation periods totaling 37 and 45 minutes.

In two animals, male *cat 10* and female *cat 15*, the nictitating membrane response failed. The protocols reveal the following: *Cat 10* had considerable hemorrhage at operation; three hours later mean arterial pressure was 80 mm. Hg. One-half minute stimulations, alternating with one-minute rest intervals, were begun. The nictitating membrane did not respond to the

Received for publication January 20, 1942.

[1] SECKER, J.: *J. Physiol.* 94: 259. 1938; Ibid. 95: 282. 1939.

[2] SWINGLE, W. W., W. M. PARKINS, A. R. TAYLOR AND H. W. HAYS: *Am. J. Physiol.* 124: 22. 1938.

[3] ARMSTRONG, C. W. J., R. A. CLEGHORN, J. L. A. FOWLER AND G. A. MCVICAR: *J. Physiol.* 96: 146. 1939.

[4] CLEGHORN, R. A., C. W. J. ARMSTRONG, D. C. AUSTEN AND G. A. MCVICAR: *Am. J. Physiol.* 132: 542. 1940.

[5] MAISON, G. L., AND A. G. BROEKER: *Am. J. Physiol.* 132: 416. 1940.

12th stimulation, just prior to which respiration had ceased and artificial measures were begun; mean arterial pressure 42 mm. Hg. Saline injection raised the pressure to a maximum of 83 mm. Hg for a time, but no nictitating membrane responses were elicited either by stimulation or by intravenous epinephrine (1 μg.). Similarly, in *cat 15*, female, the mean pressure dropped suddenly three hours postoperatively, although little blood had been lost. Locke's solution intravenously improved the pressure for a short time, but retraction of the nictitating membrane was not obtained, either by stimulation or by epinephrine administration (1 μg.), and the cat died of circulatory failure. Autopsy revealed no hemorrhage.

The failures were probably attributable to systemic factors, in view of the inefficacy of epinephrine. On the other hand, low terminal pressures were recorded in several preparations (table 1) in which no failure of response of the nictitating membrane appeared; in these, however, the pressor and nictitating-membrane responses to epinephrine persisted.

TABLE 1. RECORD OF NICTITATING MEMBRANE RESPONSES TO CERVICAL SYMPATHETIC STIMULATION IN ADRENALECTOMIZED CATS

Animal, sex	Stimulation begun[1]	Total stimulation, minutes	Blood pressure responses	Terminal blood pressures, mm. Hg	Nictitating membrane responses[2]
1, ♀	5	47			No failure (120)
2, ♂	30	12			No failure (129)
3, ♂	60	24		140	No failure (240)
4, ♂[3]	90	18	Depressor	40	No failure (275)
5, ♀	120	42			No failure (190)
7, ♂	60	95	Depressor	72	No failure (284)
8, ♂	376	7			No failure (490)
9, ♀	260[4]	37	Pressor	48	No failure (513)[4]
10, ♂	330	6	Dep-Pres	44	Failure (420)
11, ♂	200	43	Pressor	90	No failure (450)
12, ♂	202	50	Pressor	54	No failure (435)
14, ♂	165	22	Depressor	30	No failure (360)
15, ♀	200	20	No response	30	Failure (180)[4]
16, ♂	105	45	Dep-Pres	32	No failure (385)

[1] Minutes after adrenalectomy.
[2] Figures in parentheses, minutes from adrenalectomy to end of experiment.
[3] Small residual adrenal tissue found at autopsy.
[4] Oöphorectomy-adrenalectomy.

In the remaining 12 animals the nictitating-membrane mechanism showed no obvious signs of deterioration, for periods ranging to 503 minutes post-adrenalectomy and, in one case, following a total of 95 minutes of supramaximal stimulation.

The blood-pressure responses which accompanied retraction of the nictitating membrane proved variable, as indicated in the table, in that some animals showed pressor responses only, others depressor; two (*cats 10, 16*) showed depressor responses at the outset, later changing to pressor. Our cats differed from those of Secker, which gave pressor responses only, in that the contralateral vago-sympathetic trunk was left intact.

Summary. In 12 of 14 acutely adrenalectomized cats retraction of the nictitating membrane in response to stimulation of the corresponding cervical sympathetic nerve persisted for periods up to 8½ hours, despite repeated and intense stimulation. Ultimate failure in the remaining 2 animals may have been related to their unphysiological condition, since no response was obtained upon epinephrine injection. It would appear that if the adrenal cortex provides a substance concerned in formation of the neuro-myal transmitting agent involved, it is not indispensable, or is not exhausted, under the conditions of the experiments reported.

HANS O. HATERIUS
GEORGE L. MAISON

Department of Physiology
College of Medicine
Wayne University
Detroit, Michigan

THE RELATIONSHIP OF THE AMINO GROUP TO THE ACTIVITY OF PREGNANT MARE SERUM

LI, SIMPSON AND EVANS[1] showed that the primary amino groups of prolactin were important for the activity of that hormone. In a later paper[2] the same authors showed that acetylation by ketene produced inactivation of pituitary FSH, LH and PMS under conditions which permitted acetylation of the free amino groups but not of the tyrosine-hydroxyl group. Prolan[2] was not inactivated rapidly under the same conditions and the authors consider that its inactivation was due not to the action of the ketene on the primary amino groups but to the acetylation of the hydroxyl group of the tyrosine present.

HNO_2 has been shown to inactivate PMS rapidly at 0°C. in 30 minutes.[3] Formic acid at pH 8.0 for 3 hours has been shown to produce 85 per cent inactivation of PMS.[4]

Phenyl isocyanate has been used by Bottomley and Folley[5] to show that the free amino group of the lactogenic hormone is essential for its activity. They consider the reagent to be especially reliable for this purpose because the conditions used are extremely mild (0°C. and pH 8.0). Hopkins and Wormall[6] concluded that phenyl isocyanate reacts only with the free amino groups of the lysine components of the molecule.

Acetic anhydride has been shown to produce 25 per cent inactivation of prolan,[7] not to affect prolactin,[8] and to destroy completely the activity of the pituitary (I.C.S.H.).[9]

Experimental procedure. A purified fraction of PMS assaying 40 Cartland-Nelson units per milligram has been used in these studies.

Dissolve 18.8 mg. of PMS in 10 cc. of phosphate buffer, pH 7.4; add 0.04 cc. of phenyl isocyanate and place the mixture in an ice-salt bath. Shake frequently for one hour. Adjust the pH to neutrality and centrifuge off the insoluble diphenyl urea.

Dissolve 9.2 mg. of PMS in 2 cc. of water. Add 200 mg. of $NaHCO_3$ and 0.07 cc. of acetic anhydride. Remove an aliquot at the end of one hour and after 20 hours, for assay.

Assays were made by the method of Cartland and Nelson.[10] The results are given in table 1.

TABLE 1. ACTION OF REAGENTS ON THE AMINO GROUP OF PMS

Fraction and treatment	Ovarian weight	Units	Degree of inactivation
	mg.	*per mg.*	%
PMS+Acetic anhydride, 1 hour	16	2.7	93
PMS+Acetic anhydride, 20 hours	17	1.2	96
PMS+Phenyl isocyanate, 60 min.*	18	3	95
PMS+Phenyl isocyanate, 60 min.*	14	1	100
PMS (Control)	65	40	—

* Assayed at two levels.

Conclusion. The previous experimental work using HNO_2 formic acid, and ketene, has shown that the amino group is important for the activity of PMS. We have shown that the treatment of PMS by phenyl isocyanate and acetic anhydride likewise inactivates the hormone. The action of the phenyl isocyanate may be on the amino group of the lysine molecule,

Received for publication December 23, 1941.

[1] LI, C. H., M. E. SIMPSON AND H. M. EVANS: *Science* 90: 140. 1939.
[2] LI, C. H., M. E. SIMPSON AND H. M. EVANS: *J. Biol. Chem.* 131: 259. 1939.
[3] LI, C. H., H. M. EVANS AND D. WONDER: *J. Gen. Physiol.* 23: 733. 1940.
[4] CARTLAND, G. F., AND J. W. NELSON: *J. Biol. Chem.* 119: 59. 1937.
[5] BOTTOMLEY, A. C., AND S. J. FOLLEY: *Nature* 145: 304. 1940.
[6] HOPKINS, S. J., AND A. WORMALL: *Biochem. J.* 27: 740. 1933.
[7] BISCHOFF, F., AND M. L. LONG: *J. Biol. Chem.* 116: 285. 1936.
[8] MCSHAN, W. H., AND H. E. FRENCH: *J. Biol. Chem.* 117: 111. 1937.
[9] BISCHOFF, F.: *J. Biol. Chem.* 133: 621. 1940.
[10] CARTLAND, G. F., AND J. W. NELSON: *Am. J. Physiol.* 122: 201. 1938.

as suggested by Hopkins and Wormall.[6] The presence of lysine in PMS has not been demonstrated. Our results are in agreement with those of previous workers who associate the presence of free amino groups with the activity of PMS.

JOHN S. EVANS
JOHN W. NELSON
GEORGE F. CARTLAND

From the Research Laboratories
The Upjohn Company
Kalamazoo, Michigan

A METHOD OF EXTRACTION AND ASSAY OF ADRENOCORTICOTROPIC HORMONE FROM HUMAN URINE

THE ANTERIOR PITUITARY gland produces a hormone which maintains and stimulates the adrenal cortex. This adrenocorticotropic hormone was first described by Collip, Anderson and Thomson.[1] Experimental work on the pituitary-adrenocortical relationship has been reviewed recently.[2] Since this pituitary-adrenal relationship is probably of great importance in a number of clinical conditions it seemed desirable to attempt assay of adrenocorticotropic hormone. Jores[3] injected deproteinized concentrated serum into immature mice and weighed the adrenals 24 hours later. An increase of adrenal weight per 100 gm. of body weight over 27 mg. was regarded as proof of an excess of adrenocorticotropic hormone. We have used this method extensively in the study of Cushing's syndrome[4,5] and a variety of other conditions.

However, this method has several disadvantages. The yield after deproteinizing, detoxifying and concentrating the serum is very small, 50 cc. of blood yielding at best sufficient material for assay on 5 mice. Quite frequently the yield is even less. Due to variation in the weight of the adrenal glands, at least 5 mice should be used for each test. Drawing of larger amounts of blood from a patient is obviously impracticable as a routine procedure. Consequently, at the very best one dosage level on each serum specimen can be tested.

It was consequently decided to attempt to assay of adrenocorticotropic hormone in human urine.

Extraction. The method of extraction employed is a modification of the procedure of Bates and Riddle[6,7] for preparations of adrenotropic extracts from the pituitary gland. The 24-hour-urine specimen was measured and alkalized with N NaOH to PH 9–10, using phenolphthalein as indicator. One thousand cc. of 95 per cent ethyl alcohol was then added to each 400 cc. of urine, thus bringing the alcohol concentration to 70 per cent. This mixture was stirred for one hour. After centrifuging, the supernatant fluid was adjusted to PH 5.5 to 6 with N HCl, using bromthymol blue as indicator. Two to three volumes of ethyl alcohol were added, bringing the final alcohol concentration to at least 86 per cent. This preparation is kept in the refrigerator over-night, at which time a fine precipitate has appeared. The supernatant fluid was decanted and the precipitate dried and stored in the refrigerator until it was tested. For assay, solutions were made in distilled water. In several instances N NaOH had to be added in order to secure solution. These solutions were adjusted to PH 5.5 to 6 (bromthymol blue) with N HCl.

Assay methods. Extracts were assayed on 2-day-old chicks[7] and on 4-day-old rats.[8]

Received for publication February 4, 1942.

[1] COLLIP, J. B., E. M. ANDERSON AND D. L. THOMPSON: *Lancet* 2: 397. 1933.
[2] SWANN, H. G.: *Phys. Rev.* 20: 493. 1940.
[3] JORES, A.: *Klin. Wchnschr.* 1925: 14. 1938.
[4] PASCHKIS, K.: *J. Clin. Investigation* 19: 776. 1940.
[5] RAKOFF, A. E., A. CANTAROW AND K. E. PASCHKIS: *J. Clinical Endocrinology* 1: 912. 1941.
[6] BATES, R. W., AND O. RIDDLE: *J. Pharmacol. & Exper. Therap.* 55: 365. 1935.
[7] BATES, R. W., O. RIDDLE AND R. A. MILLER: *Endocrinology* 27: 781. 1940.
[8] MOON, H. D.: *Proc. Soc. Exper. Biol. & Med.* 43: 42. 1940.

The adrenals of rodents increase in weight under the influence of various toxic agents as well as under the influence of adrenocorticotropic hormone. The specificity of results obtained with crude extracts in rats or mice is, therefore, open to doubt. The adrenals of chicks have been shown to be very much less sensitive to such toxic stimuli.[7] Weight increase of the adrenals in this species can, therefore, with all probability be considered to be due to adrenocorticotropic hormone.

The chick assay was performed on white Leghorn chicks procured from a poultry farm. The procedure published by Bates, Riddle and Miller[7] was followed with minor variations introduced in order to adapt it to the routine of our laboratories. Chicks were received on Mondays, injected on 5 consecutive days and killed on the seventh day. The adrenals were dissected and weighed immediately on a torsion balance. Injections were given in single daily dose or in 3 divided daily doses. Six test animals and 6 controls were used for each dosage level.

Rat assays were performed on 4-day-old litters of our own colony, originated from Wistar rats. Half of each litter was used as controls, care being taken to divide males and females equally between experimental and control animals. Rats were injected intraperitoneally on 3 subsequent days and killed on the fourth day. Fresh adrenal weights were determined on the torsion balance.

Dosage. Several dosage levels were employed in chicks. Significant increase of adrenal weights was obtained in a number of cases with 5 daily doses of 8 to 10 mg., totaling 40 to 50 mg. We as yet have not observed an output of adrenocorticotropic hormone high enough to show increase of adrenal weights with a total dosage of 25 mg. In several instances a total dosage of 100 mg. per chick was given. Since Bates, Riddle and Miller have described a linear relationship of dosage and response for adrenotropic pituitary extracts, it was expected that the increase of the adrenal weight (in % of the normal controls) would be twice that of the animals receiving 50 mg. This however was not the case. A total dosage of 100 mg. was toxic with some extracts, and all of the animals died. Other extracts were not toxic, permitting survival of the animals and not producing loss of body weight, but the adrenals failed to show increase over those from animals receiving the 50 mg. dose. Further purification of our extracts is now under way and will show whether this lack of increased response is due to some toxic agent in the rather crude extracts hitherto employed. At present we are performing the assay with a total dosage of 50 mg. In the assays on the 4-day-old rat we have tentatively used a total dosage of 9 mg., 3 intraperitoneal injections of 3 mg. in 0.2 cc. water.

Results. The amounts of hormone excreted vary considerably. In a number of urines no adrenocorticotropic activity was found at the dosage levels employed. The highest excretion observed so far was about 100 chick units (Bates-Riddle units) per 24 hours. A number of urines contained 10 to 20 chick units per 24 hours.

The output of adrenocorticotropic hormone is being studied in Cushing's syndrome, hirsutism, pituitary adenoma, certain types of hypertension and of diabetes, toxemia of pregnancy of other conditions. Results will be published at a later date.

Summary. A method of extracting and assaying human urine for adrenocorticotropic hormone content is described.

K. E. PASCHKIS*
A. E. RAKOFF
A. CANTAROW

From the Departments of Medicine, of Physiology and of Obstetrics, The Jefferson Medical College and the Departments of Medicine and of Obstetrics, Jefferson Hospital, Philadelphia, Pennsylvania

---

* J. Ewing Mears Fellow in Physiology and Medicine.